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Human glioblastomas overexpress ADAMTS-5 that degrades brevican

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Abstract Selective cleavage of the Glu^{395}-Ser^{396} bond of brevican, one of the major proteoglycans in adult brain tissues, is thought to be important for glioma cell invasion. Our previous biochemical study demonstrated that ADAMTS-4, a member of the ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family, has such an activity. In the present study, we examined brevican-degrading activities of ADAMTS-1, -4 and -5 at the cellular level, and their expression and localization in human glioma tissues. In 293T transfectants expressing ADAMTS-4 or ADAMTS-5, brevican was cleaved into two major fragments in an identical pattern, but no such degradation was observed with ADAMTS-1 transfectants. When the expression levels of these ADAMTS species were examined by real-time quantitative PCR, only ADAMTS-5 was found to be overexpressed in glioblastoma tissues compared to control normal brain tissues ($P < 0.05$). In situ hybridization and immunohistochemistry demonstrated that ADAMTS-5 is expressed predominantly in glioblastoma cells. Forced expression of ADAMTS-5 in glioma cell lines stimulated cell invasion. These results demonstrate for the first time that ADAMTS-5 is capable of degrading brevican and is overexpressed in glioblastoma cells, and suggest that ADAMTS-5 may play a role in glioma cell invasion through the cleavage of brevican.

Keywords ADAMTS · Glioma · Invasion · Brevican · Digestion

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Introduction

Brevican, a member of the lectican family, is one of the most abundant proteoglycans in normal adult brain tissues, and is thought to form lattice structures by linking hyaluronan and tenascin-R through the N- and C-terminal globular domains of brevican, respectively [16]. Since the brain extracellular matrix (ECM) contains no collagen fibrils, the matrix of hyaluronan/brevican/tenascin-R is considered to be essential to maintain the integrity of the brain ECM. Thus, the degradation of brevican by proteinases causes disruption of the ECM structures, which may facilitate glioma cells to invade the surrounding normal brain tissues [16]. Brevican can be degraded by many proteinases, but the major cleavage site observed under physiological conditions [15] and during glioma invasion [17] is the Glu^{395}-Ser^{396} bond present within the central domain of the core protein, forming an ~50-kDa N-terminal fragment. Interestingly, the expression of brevican is dramatically upregulated in glioma tissues [2] [3]. Forced expression of this N-terminal fragment of brevican renders noninvasive glioma cells invasive in vivo, while expression of full-length brevican has no such effect [17]. Therefore, proteinases that yield the N-terminal fragments through the cleavage of the specific site are implicated in glioma cell invasion [17].

Our previous biochemical study [12] and that of Matthews et al. [7] demonstrated that a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS-4) selectively attacks the Glu^{395}-Ser^{396} bond of brevican and generates 56-kDa N-terminal and an

83-kDa C-terminal fragments, whereas matrix metalloproteinases digest brevican into a few fragments by preferentially cleaving the Ala^{360}-Phe^{361} bond. Among the ADAMTS gene family, ADAMTS-1, -4 and -5 are known to digest aggrecan, another member of the lectican family, by cleaving the Glu-X bonds [1, 6, 13, 14]. Thus, these ADAMTS species may play a key role in promoting glioma invasion by degrading brevican in the brain ECM. However, little is known about the proteinase actions of these ADAMTS species on brevican at the cellular level, and no information is available for their expression or localization in the human glioma tissues. To address these issues, we examined the susceptibility of brevican to ADAMTS-1, -4 and -5, their expression levels, localization and function in human glioma tissues.

Materials and methods

Expression of ADAMTS-1, -4 and -5 and immunoblotting

Expression plasmid of ADAMTS-1 tagged with FLAG epitope at the C terminus was provided by Dr. Kouji Kuno (Cancer Research Institute, Kanazawa University, Ishikawa, Japan) [5]. Expression plasmids for ADAMTS-4 and ADAMTS-5 were constructed as follows: cDNA fragments encoding ADAMTS-4 or ADAMTS-5 were PCR-amplified with human glioma cell cDNA library as a template, and inserted into 3'-FLAG-pEAK plasmid. 293T cells plated onto 12-well microtiter plates were transfected with the expression plasmids for ADAMTS-1, -4 or -5 by the calcium phosphate method. The cells were harvested at 24 h after transfection and lysed in 50 µl sodium dodecyl sulfate (SDS) sample buffer containing 2% 2-mercaptoethanol. Immunoblotting was performed using anti-FLAG M2 antibody as previously described (Sigma, St. Louis, MO) [9].

Preparation of brevican and its degradation by transfectants expressing ADAMTS-1, -4 or -5

Brevican was purified from culture media of the stable transfectants expressing rat brevican using affinity column chromatography [8], and labeled with Alexa Fluor 488 Monoclonal Antibody Labeling Kit (Molecular Probe, Eugene, OR) according to the manufacture's protocol. 293T cells transfected with the expression plasmids for ADAMTS-1, -4 or -5 (100 ng/well) were incubated with Alexa Fluor 488-labeled brevican (200 ng/well) for 6 h at 37°C in DMEM (20 µl/well). After termination of the reaction with 20 mM EDTA, the media containing labeled brevican were subjected to SDS-PAGE under reducing conditions, and the digestion products were detected by Fluoroimager (Molecular Dynamics, Sunnyvale, CA).

Samples of brain tissues

Fresh human brain tumor tissues were resected from 53 patients with astrocytic tumor (9 low-grade astrocytomas, 8 anaplastic astrocytomas and 36 glioblastomas) and 9 patients with metastatic brain tumor (metastatic lung adenocarcinoma), who underwent therapeutic removal of the brain tumors. Normal brain tissues were obtained from 19 patients undergoing temporal lobectomy for epilepsy. Histological diagnosis was made by standard light microscopic evaluation of the sections stained with hematoxylin and eosin. Astrocytic tumors were classified according to the revised WHO criteria for tumors of the central nervous system [4]. All the tumor tissues were obtained at primary resection, and none of the patients had been subjected to chemotherapy or radiation therapy before operation. Median age and gender of the patients per tumor type are shown in Table 1. For the experimental use of the surgical samples, informed consent was obtained from each patient according to the hospital ethical guidelines.

Real-time quantitative PCR

Total RNA was extracted from 53 astrocytic tumors and 19 normal brain tissues by the acid guanidinium-phenol-chloroform method and incubated with DNase I (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. The PCR primers and TaqMan fluorogenic probes for ADAMTS-1, -4 and -5 (GenBank accession numbers AF207664, AF148213 and

Table 1 Clinical pathological features and mRNA levels of ADAMTS-1, -4 and -5 in astrocytic tumors and metastatic brain tumors

Histology	*n*	Age (years) (Mean ± SD)	Male/female	ADAMTS		
				−1	−4	−5
Normal brain	19	32.4 ± 15.2	10/9	0.43 ± 0.17	0.31 ± 0.18	0.15 ± 0.09
Astrocytoma						
Low grade	9	41.2 ± 14.6	4/5	0.39 ± 0.31	0.23 ± 0.24	0.16 ± 0.13
Anaplastic	8	48.1 ± 15.3	3/5	0.26 ± 0.14	0.31 ± 0.24	0.16 ± 0.16
Glioblastoma	36	58.2 ± 12.3	17/19	0.32 ± 0.32	0.32 ± 0.29	0.30 ± 0.26
Metastasis	9	62.4 ± 10.5	5/4	0.13 ± 0.12	0.16 ± 0.11	0.16 ± 0.11

NM_007038, respectively) were designed using the Primer Express software program (version 1.0, Perkin-Elmer), and specificity of the sequences was confirmed according to the methods described previously [10]. The sequences of primers and probes were as follows: ADAMTS-1, forward primer 5'-AAAGGACAGGTGCAAGCTCATC-3', reverse primer 5'-ATCTACAACCTTGGGC TGCAAA-3' and TaqMan probe 5'-AGCCAAAGGCA TTGGCTACTTCTTCG-3'; ADAMTS-4, forward primer 5'-TCAGCCTTCACTGCTGCTCAT-3', reverse primer 5'-GCCCATTCAAACTGATGCATG-3' and TaqMan probe 5'-AACTGGGTCATGTCTTCAA-CATGCTCCA-3'; ADAMTS-5, forward primer 5'-AT-CTGCCTGTGACTTTCCCTG-3', reverse primer 5'-TGTGGACAATGGCGTGAGT-3' and TaqMan probe 5'-AAGGACTATGATGCTGACCGCCAGTG-3'. The quantification of mRNA levels was performed at least three times for each sample by the ABI-PRISM 7700 sequence detection system and software (Applied Biosystems, Foster City, CA), and the level was expressed as a molar ratio to glyceraldehyde-3-phosphate dehydrogenase mRNA level [10].

In situ hybridization

Oligonucleotide DNA complementary to the mRNA transcript of ADAMTS-5 TAGAGCAGGCGAGCCA TGGGCCCGTGACCC was designed as the antisense probe for in situ hybridization. Specificity of the probe was confirmed according to the methods described previously [10]. A poly-d$(T)_{20}$ oligonucleotide was utilized to verify the integrity and lack of mRNA degradation in the samples. As for a negative control, a poly-d$(A)_{20}$ oligonucleotide was used. All the DNA probes were synthesized with six biotin molecules at the 3' end via direct coupling by standard phosphoramidite chemistry (Research Genetics, Huntsville, AL). In situ hybridization was performed on paraffin sections of paraformaldehyde-fixed normal brain and glioblastoma tissues using the microprobe manual staining system (Fisher Scientific, Pittsburgh, PA) [10].

Immunohistochemistry

Paraffin were treated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity and then microwaved for 5 min at 500 W. After blocking nonspecific binding with 10% goat serum, they were reacted with rabbit anti-ADAMTS-5 polyclonal antibodies (1:100 dilution; Triple Point Biologics, Inc. Forest Grove, OR) or rabbit non-immune IgG (1:100 dilution; Vector Laboratories, Burlingame, CA). Subsequently, the specimens were incubated with biotinylated goat anti-rabbit IgG (DakoCytomation, Carpinteria, CA), followed by the peroxidase-labeled avidin:biotin complex method (1:500 dilution; Amersham Biosciences, Piscataway, NJ). After the reactions, the sections were counterstained with hematoxylin.

Cell transfection and invasion assay

Human astrocytoma cell lines U87, U251 and T98G, (American Type Culture Collection, Manassas, VA) were maintained in DMEM supplemented with 10% fetal bovine serum. Transient transfection was performed into these glioma cell lines by Effectene (Qiagen, Valencia, CA) as recommended by the manufacturer's protocol. Cells transfected with the empty plasmid vector were used as controls. Transfected cells were cultured for an additional 48 h prior to use. Cell invasion assays were carried out using modified Boyden chambers consisting of Transwell membrane filter inserts in 24-well tissue culture plates (BD Biosciences Discovery Labware, Bedfold, MA) as described previously [11]. The top surfaces of the Transwell membranes were coated with 1 mg/ml Matrigel basement membrane matrix (Becton Dickinson Labware) mixed with brevican (200 ng) overnight at 4°C. Serum-deprived cells (2×10^5) suspended in 100 µl DMEM containing 1 mg/ml BSA, and 0.5% serum were added to each Transwell. After 16 h, noninvading cells were removed by wiping the upper side of the membrane, and the invaded cells were fixed with methanol and stained using crystal violet (Sigma). The number of invaded cells was quantified by counting them in at least six random fields (×200 of total magnification) per filter.

Statistics

Statistical analyses were performed using the two-tailed Mann-Whitney U test. $P<0.05$ was considered significant.

Results

Degradation of brevican by transfectants expressing ADAMTS-1, -4 or -5

The expression of ADAMTS-1, -4 and -5 in 293T cells transfected with the plasmids for each ADAMTS species was confirmed by immunoblotting using anti-FLAG M2 antibody. As shown in Fig. 1A, protein bands of ADAMTS-1 of 90 kDa, ADAMTS-4 of 69 kDa and ADAMTS-5 of 74 kDa were detected, while 293T cells transfected with the expression vector alone (pEAK) showed no band. The molecular masses corresponded to those of the active ADAMTS species [1, 5, 12]. When these transfectants were cultured in the presence of Alexa Fluor 488-labeled brevican, the

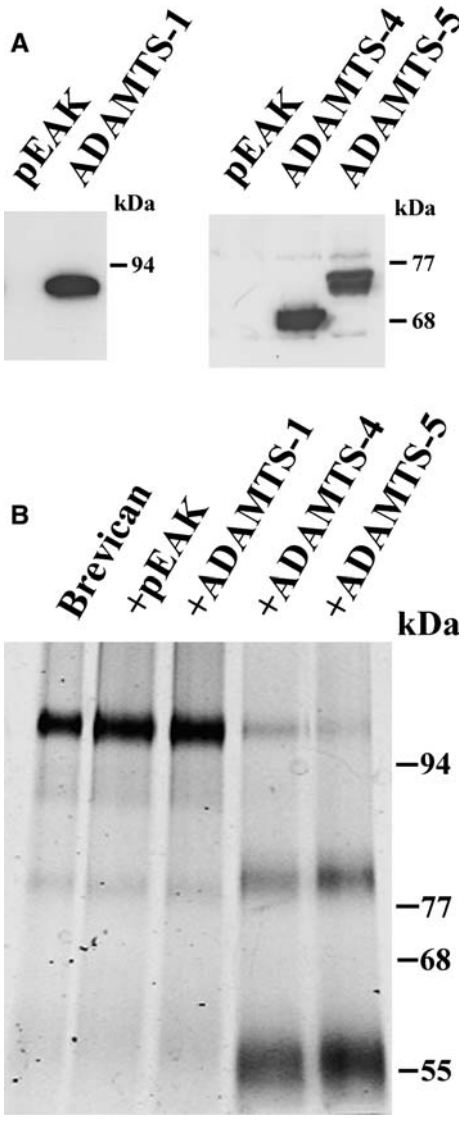

Fig. 1 Expression of ADAMTS-1, -4 and -5 and digestion of brevican by their transfectants. **A** Immunoblotting of ADAMTS-1, -4 and -5 in 293T cells transfected with vector alone (pEAK) or expression plasmids containing ADAMTS-1, -4 or -5 cDNA. Cell lysates were immunoblotted with anti-FLAG M2 antibody. **B** Brevican digestion by 239T transfectants expressing ADAMTS-1, -4 or -5. Alexa Fluor 488-labeled brevican was incubated with the ADAMTS transfectants or mock transfectants (pEAK). The digestion products were analyzed by SDS-PAGE and monitored by Fluoroimager. Brevican denotes the control brevican sample incubated with buffer alone

transfectants expressing ADAMTS-4 or ADAMTS-5 produced two major fragments of 83 and 56 kDa in an identical pattern (Fig. 1B). However, ADAMTS-1 transfectants or mock-transfectants showed no degradation products (Fig. 1B). The brevican digestion by ADAMTS-4 or ADAMTS-5 transfectants was abolished in the calcium-free culture medium (data not shown), indicating that the digestion was dependent on metalloproteinase activity.

Overexpression of ADAMTS-5 in human glioblastomas

To evaluate the expression levels of ADAMTS-1, -4 and -5, the tissue samples were subjected to real-time quantitative PCR. As shown in Fig. 2 and Table 1, there was no significant difference in the levels of ADAMTS-1 among control normal brains and astrocytic tumors. A similar expression pattern was observed with ADAMTS-4. On the other hand, the expression of ADAMTS-5 in glioblastomas was remarkably approximately twofold higher than that in normal brains ($P < 0.05$) (Fig. 2, Table 1). The level also tended to be higher than those in low-grade astrocytomas, anaplastic astrocytomas and metastatic brain tumors, although there were no significant differences (Fig. 2, Table 1).

Determination of cells expressing ADAMTS-5 in glioblastoma and normal brain tissues

Since ADAMTS-5 was overexpressed in glioblastomas compared to low-grade astrocytomas, anaplastic astrocytomas and normal brains, cells expressing ADAMTS-5 gene in glioblastomas and control normal brains were determined by in situ hybridization and immunohistochemistry. The poly-d$(T)_{20}$ probe gave a strong signal in all the cells of glioblastoma and control brain tissues (Fig. 3A, B), but only a background signal was obtained with the poly-d$(A)_{20}$ probe (Fig. 3C, D). These findings indicate that mRNAs within the cells in the tissues are well conserved, and the sections used are suitable for in situ hybridization. As shown in Fig. 3E, strong signals for ADAMTS-5 mRNA were detected with the antisense RNA probe predominantly in the neoplastic glioblastoma cells. On the other hand, most cells in the normal brain tissues showed negligible signals, but some neurons were positive (Fig. 3F). By immunohistochemistry, ADAMTS-5 was localized mainly to the glioblastoma cells (Fig. 4C). Invading neoplastic cells also contained significant staining for ADAMTS-5 (Fig. 4A, B). Endothelial cells of blood vessels in the glioblastoma tissues and reactive astrocytes were occasionally immunostained positively for ADAMTS-5. In normal brain tissues, neurons were immunoreactive (Fig. 4E). However, no staining was seen, when the primary antibody was replaced with non-immune IgG in glioblastoma (Fig. 4D) or normal brain tissues (Fig. 4F). These findings are consistent with the data of in situ hybridization and real-time quantitative PCR analyses.

Induction of cell invasion by ADAMTS-5

To examine functional effects of ADAMTS-5, U87, U251 and T98G cells transfected with ADAMTS-5 or control vector (pEAK) constructs were evaluated in invasion assay. Immunoblotting data showed that ADAMTS-5 protein was produced in transfected cells (Fig. 5A). These cells grew with comparable doubling times as

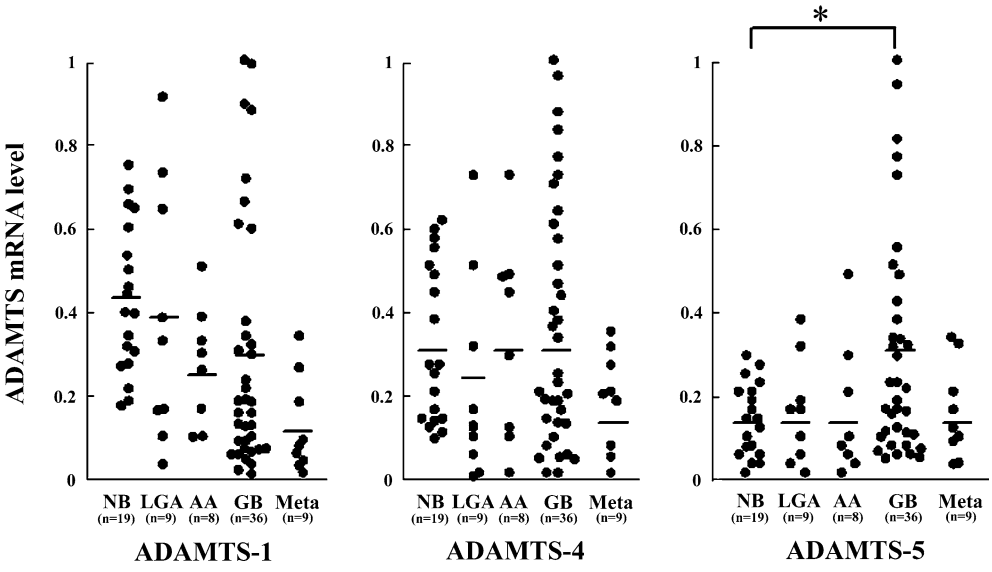

Fig. 2 The mRNA expression levels of ADAMTS-1, -4 and -5 in normal brain and brain tumor tissues. Relative mRNA expression levels of ADAMTS-1, -4 and -5 in normal brains (*NB*), low-grade astrocytomas (*LGA*), anaplastic astrocytomas (*AA*), glioblastomas (*GB*) and metastatic brain tumors (*Meta*) were analyzed by real-time quantitative PCR. The level is expressed as a proportion to the highest mRNA level of each ADAMTS, which was given a value of 1. *Bars* indicate mean values. $*P < 0.05$

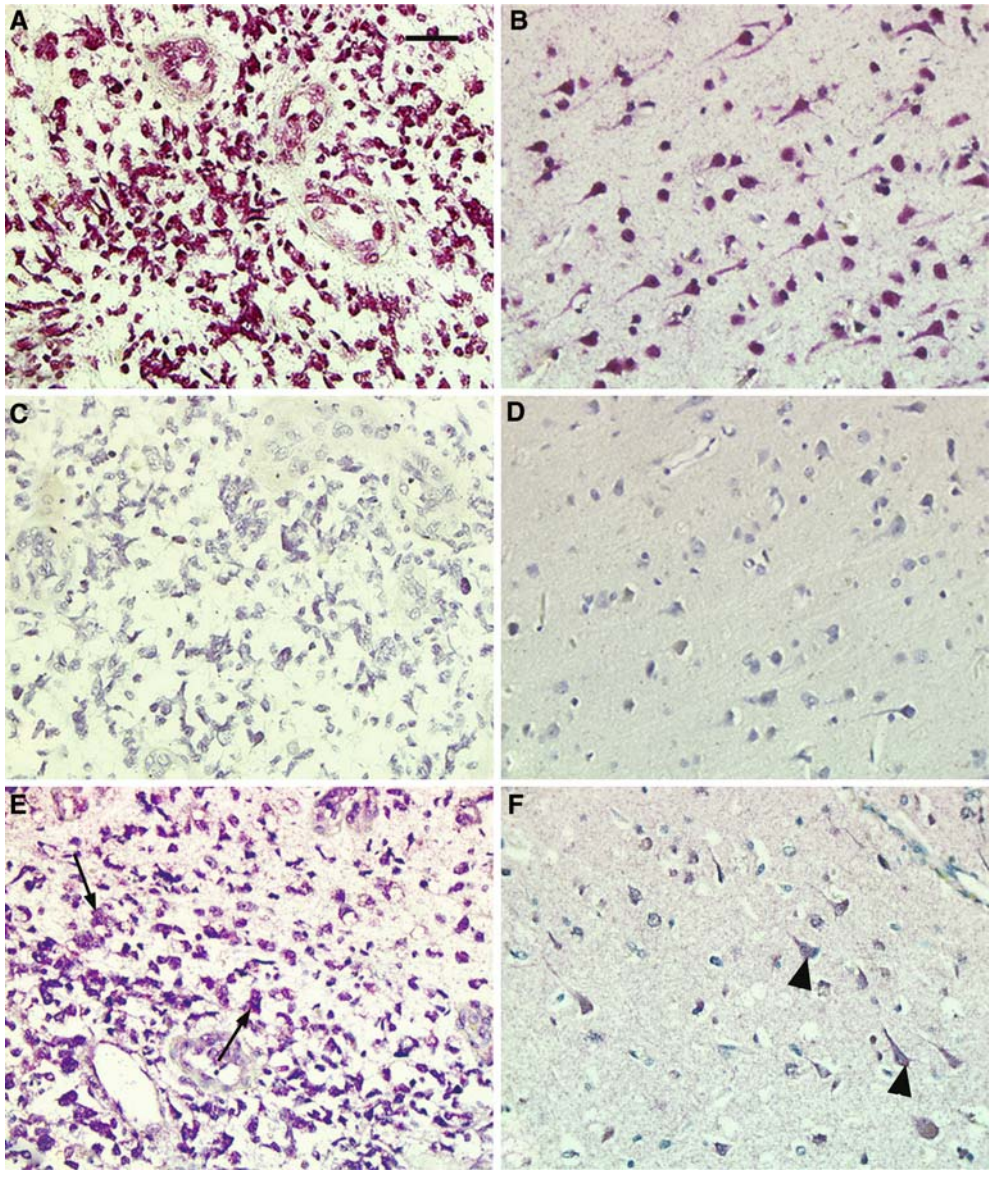

Fig. 3 In situ hybridization of ADAMTS-5 in glioblastoma and normal brain tissues. Paraffin sections of glioblastoma (**A, C**, **E**) and normal brain tissues (**B, D**, **F**) were reacted with a poly-d$(T)_{20}$ probe (positive control) (**A**, **B**), a poly-d$(A)_{20}$ probe (negative control) (**C**, **D**) or antisense oligonucleotide DNA probe for ADAMTS-5 (**E**, **F**). *Arrows,* glioblastoma cells with a positive reaction; *arrowheads*, neurons with a positive reaction. Hematoxylin counterstain. *Bar* 50 μm

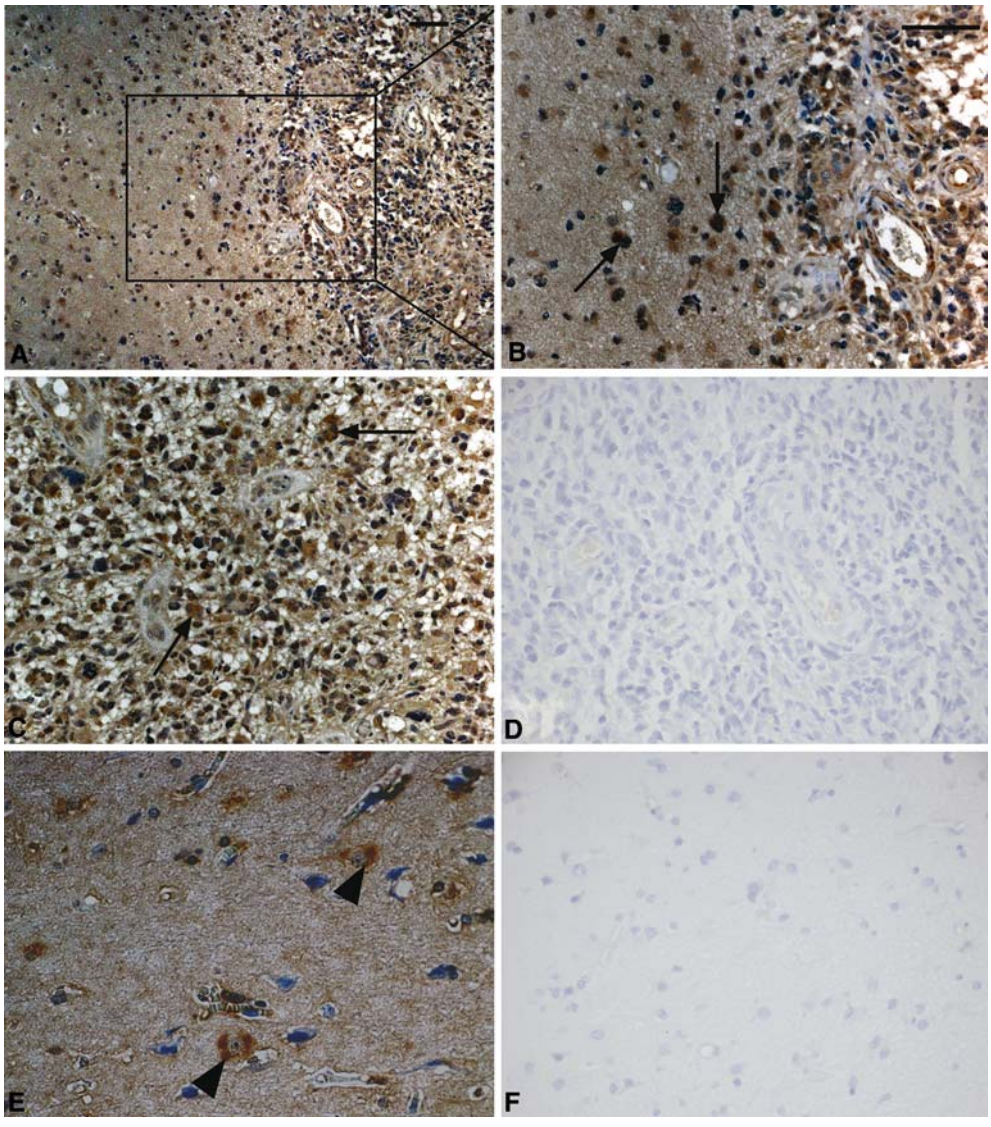

Fig. 4 Immunolocalization of ADAMTS-5 in glioblastoma and normal brain tissues. Paraffin sections were immunostained with polyclonal antibodies against ADAMTS-5 (**A** invading front in low magnification**, B** high magnification, **C** tumor core, **E** normal brain) or non-immune rabbit IgG (**D** tumor core, **F** normal brain) as described in Materials and methods. Note the ADAMTS-5 immunostaining in the glioblastoma cells at the central region in the tumor (**C**, *arrows*), as well as in invading glioblastoma cells at the tumor border (**B**, *arrows*). ADAMTS-5 is also immunostained in neurons (*arrowheads* in **E**). No staining is observed with non-immune IgG (**D**, **F**). Hematoxylin counterstain. *Bar* 50 μm

the parental cells; cells expressing exogenous ADAMTS-5 did not display any cytotoxic effects. As shown in Fig. 5B, cell invasion through membranes coated with Matrigel containing brevican was increased in cells expressing ADAMTS-5 (1.4- to 2.7-fold) in all cell lines compared to the control pEAK transfectant, indicating that the ADAMTS-5 induces glioma cell invasion.

Discussion

In the present study we have demonstrated that among the three ADAMTS species expressed in the adult brain, ADAMTS-4 and ADAMTS-5 cleave brevican into two major fragments of 56 and 83 kDa. The digestion products obtained in the present experiment were similar to the fragments generated through the specific cleavage of the Glu^{395}-Ser^{396} bond of brevican by the action of purified recombinant ADAMTS-4 [7] [12]. Therefore, the data not only support the previous biochemical finding on brevican digestion by ADAMTS-4 [7, 12], but also demonstrate that the cleavage by ADAMTS-4 occurs at the cellular level. Importantly, an identical digestion pattern was observed by incubation with transfectants expressing ADAMTS-5. Accumulated lines of evidence have indicated that ADAMTS-4 and ADAMTS-5 share substrate specificity by attacking aggrecan at the five identical Glu-X bonds in the aggrecan core protein [14]. Thus, it is conceivable that like ADAMTS-4, ADAMTS-5 cleaves the Glu^{395}-Ser^{396} bond of brevican. ADAMTS-1 is known to digest aggrecan core protein by cleaving the Glu^{1871}-Leu^{1872} bond that is also susceptible to ADAMTS-4 and ADAMTS-5 [6]. However, contrary to our expectation, brevican was resistant to ADAMTS-1 expressed by transfectants.

Brevican is thought to play a key role in maintaining the integrity of the brain ECM through molecular bridges with hyaluronan and tenascin-R [16]. Disruption of the bridges by proteolytic cleavages should facilitate glioma cell invasion [16]. Previous studies have shown

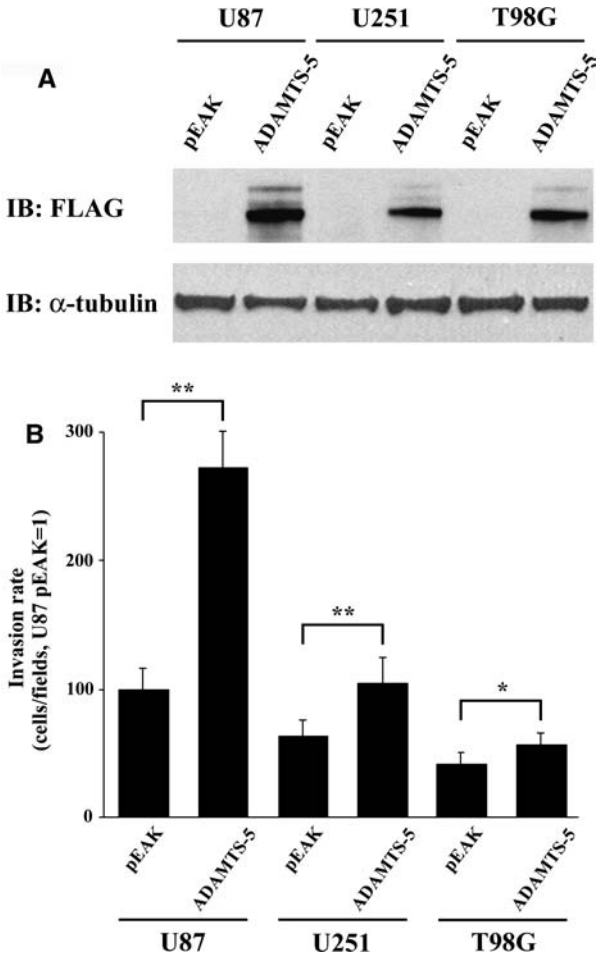

Fig. 5 Cell invasion of glioma cell lines expressing ADAMTS-5. **A** Cells were transfected with ADAMTS-5 or pEAK, and cell lysates were then immunoblotted with FLAG M2 antibody or α-tubulin. **B** Transfected cells were applied to the invasion assay. Mean cell counts from at least six fields and four experiments are shown. *Bars* SE, $*P < 0.05$; $**P < 0.01$ vs pEAK in each cell line

that brevican is up-regulated in glioma tissues [2, 3]. In addition, overexpression of the ~50-kDa N-terminal fragments of brevican is associated with an invasive phenotype of glioma cells [17], although its molecular mechanism has not been clarified. Thus, proteinases responsible for brevican degradation are considered to be important for the glioma cell invasion [17]. In the present study, we have provided the first evidence that, although both ADAMTS-4 and ADAMTS-5 can generate such brevican fragments, only ADAMTS-5 is overexpressed in human glioblastomas. Our in situ hybridization and immunohistochemistry demonstrated that ADAMTS-5 is overexpressed predominantly by glioblastoma cells, also by invading cells. In addition, overexpression of ADAMTS-5 increased invasion of glioma cells as measured with in vitro invasion assay using Matrigel mixed with brevican. Therefore, our data suggest that ADAMTS-5 is functionally involved in glioma invasion in vivo through degradation of brevican. On the other hand, the constitutive expression of ADAMTS-4 in normal brain tissues suggests its role in the physiological turnover of brevican.

In summary, we have demonstrated that ADAMTS-5 is capable of digesting brevican in an identical pattern to ADAMTS-4, and is overexpressed by glioblastoma cells. Our data suggest the possible implications of ADAMTS-5 in human glioblastoma cell invasion.

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