**ABSTRACT**

Testican family proteins are putative extracellular heparan/chondroitin sulfate proteoglycans of unknown function. We identified recently N-Tes, which is a product of testican 3 splicing variant gene, as an inhibitor of membrane-type matrix metalloproteinases (MT-MMPs). The inhibitory function is common among testican family members except for testican 2, which was shown to uniquely abolish inhibition of MT1-MMP- or MT3-MMP-mediated pro-MMP-2 activation by other testican family members. Testican 2 inactivates N-Tes by binding to the COOH-terminal extracellular calcium-binding domain of N-Tes through its NH₂-terminal unique domain as demonstrated by communoprecipitation analysis, and, thus, testican 2 was unable to inactivate a N-Tes deletion mutant lacking the extracellular calcium-binding domain (N-TesΔ122). Migration of U251 cells on collagen, which was dependent on MT1-MMP activity under serum-free condition, was inhibited by N-Tes or N-TesΔ122 deposited on collagen. Testican 2 was not incorporated into collagen by itself, and was deposited only in the presence of N-Tes, suggesting that testican 2 binds to N-Tes deposited on collagen. Binding of testican 2 to N-Tes deposited on collagen allowed migration of cells expressing MT1-MMP. Unlike wild-type N-Tes, N-TesΔ122 did not bind to testican 2, and, thus, expression of testican 2 did not recover cell migration blocked by N-TesΔ122. In situ hybridization showed that neurons are a major source of all of the testican family members in the normal brain. The quantitative reverse transcription-PCR analysis demonstrated that all of the testican family members are expressed prominently in normal brain, and their expression levels decrease as tumor grade increases. The expression level of testican 2 was the highest among testican family members regardless of histological grade of astrocytic tumors. These results suggest that abundant distribution of testican 2 may contribute to glioma invasion by inactivating other testican family members including N-Tes, which all inhibit MT-MMPs. We propose that N-TesΔ122, which is resistant to testican 2, may have therapeutic potential as a barrier against glioma invasion.

**INTRODUCTION**

MMPs are a family of Zn²⁺–dependent enzymes that are essential for ECM turnover in normal and pathological conditions (1–3). To date, 21 mammalian MMPs have been identified by cDNA cloning, and they can be subgrouped into 15 soluble-type and 6 MT-MMPs. MMPs are overexpressed in various human malignancies (2, 3). The first MT-MMP (MT1-MMP) was identified as an activator of pro-MMP-2, which functions on the cell surface (4). Although the proteolytic activation of pro-MMP-2 is a common functional role proposed for all of the MT-MMPs (4–8), expression of MT1-MMP most closely correlates with invasive phenotype of human tumors (9–13).

Testican was first defined as an unnamed chondroitin/heparan sulfate proteoglycan in seminal plasma (14). Corresponding cDNAs were isolated from human testis libraries, and the deduced protein was named testican (15). To date 3 members of the testican family were identified by cDNA cloning, with deduced amino acid homologies of 42%, 51%, and 44% for testican 1/testican 2, testican 1/testican 3, and testican 2/testican 3, respectively (15–17). Testican 1 and testican 2 are extracellular proteoglycans highly expressed in brain, and it was expected that testicans might contribute to the proteoglycan-rich ECM of the brain and associate with neurogenesis (17–19). However, the biological function of testicans has not been extensively explored. We identified recently a splicing variant of the testican 3 gene by the expression cloning method, the product of which inhibits pro-MMP-2 processing mediated by MT-MMPs (20). Furthermore, we revealed that all members of testican except testican 2 interfered with pro-MMP-2 activation mediated by MT1-MMP or MT3-MMP (20). This suggests that testicans may regulate ECM degradation by interacting with MT-MMPs. However, its biological role in controlling ECM turnover has not been explored thus far. In addition, the expression level and tissue localization of testican mRNA in tumor tissues have not been studied.

In this study, we examined the interaction between testican 2 and other testican family proteins, and demonstrated that testican 2 abolishes inactivation of MT-MMPs by other testican family, and permits migration of glioma cells expressing MT1-MMP in the presence of other testican family proteins. Furthermore, we showed that testican 2 was expressed more highly than other testican family members both in normal and malignant astrocytic tissues. These results suggest that in contrast to other testican family members, only testican 2 contributes to malignant behavior of astrocytic tumors.

**MATERIALS AND METHODS**

**Reagents.** DMEM was from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Primers were synthesized by Genset (Kyoto, Japan). Anti-FLAG M2 and anti-HA antibodies were purchased from Sigma and Santa Cruz Biotech (Santa Cruz, CA), respectively.

**Cell Culture.** Human embryonic kidney 293T cells, and U251, U87, U373, and T98G glioma cells were obtained from Health Science Researches Bank (Osaka, Japan) and cultured in DMEM supplemented with 10% fetal bovine serum.

**Expression Plasmids.** Expression plasmids for MT1-MMP, MT3-MMP, MMP-2, and TIMP-2 were as described previously (4, 21). Expression plasmids for testican 1, testican 2, testican 3, N-Tes, and N-Tes/testican-2 chimeric proteins tagged with FLAG or HA epitope at the COOH terminus were constructed as described previously (20, 22). Expression plasmids for N-TesΔ122, testican 2–Δ133, and testican 2–Δ89 tagged with FLAG or HA epitope were constructed by inserting cDNA fragment into respective plasmids. N-TesΔ122 cDNA fragment was PCR-amplified with pEAK5 8' primer and N-Tes reverse primer TCTAGATCCGCTTCTTTCATCTGT that starts at nucleotide 473 (GenBank accession no. AB054866) with an extra Xba I site (italicized). The cDNA fragments encoding testican 2–Δ133 and testican 2–Δ89 were amplified with testican 2 forward primer GAATTCAGACCCAGAT-3364

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Received 9/5/02; accepted 4/15/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by grants-in-aid for young scientists (B-14770707 to M. N.) and for scientific research (21370053 to H. M.) from the Ministry of Education, Science and Culture of Japan. The Ministry of Education, Science, Culture and Sport of Japan. The Ministry of Education, Science, Culture and Sport of Japan.

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4 The abbreviations used are: MMP, matrix metalloproteinase; ECM, extracellular matrix; TIMP, tissue inhibitor of metalloproteinase; MT, membrane type; HA, influenza virus hemagglutinin; EC, extracellular C2–binding; RT-PCR, reverse transcription-PCR; GADPH, glyceraldehydes-3-phosphate dehydrogenase; ISH, in situ hybridization; N-, NH₂-.
Each probe was confirmed as described previously (25). All of the DNA probes signed complementary to the mRNA transcripts (Table 2). The specificity of the assays was confirmed by washing with 0.025% EDTA, U251 cells stably expressing MT1-MMP were transfected with plasmids for testican tagged with FLAG epitope or TIMP-2 (450 ng) as indicated. The culture supernatants were subjected to gelatin zymography (top panel). Cell lysates were analyzed by Western blotting with anti-FLAG antibody (bottom panel). R expression plasmids for MMP-2 (50 ng), MT3-MMP (100 ng), and N-Tes-FLAG (450 ng) were cotransfected with testican 2-FLAG plasmid for N-Tes-FLAG, N-Tes–122-FLAG, and/or testican 2-HA were expressed as a molar ratio to GAPDH mRNA level. The classification of human brain tumors had been subjected to chemotherapy or radiation therapy before resection. The tumor tissues were obtained at primary resection, and none of the patients had undergone any therapeutic intervention.

**RESULTS**

**Testican 2 Abolishes Inactivation of MT-MMPs by Other Testicans.** Testican family members, except for testican 2, inhibit pro-MMP-2 processing mediated by MT1-MMP or MT3-MMP by binding to them (20). The effect of testican 2 expression on the inhibition of MT-MMPs by other testican family proteins was studied. Pro-MMP-2 was processed to an active form (Mr 62,000) through an intermediate form (Mr 64,000) by MT1-MMP, and mainly to an intermediate form by MT3-MMP (Fig. 1A). Pro-MMP-2 processing was abolished by testican 2, while other testican family proteins acted as inhibitors. Gelatin zymography, Western blotting, and Immunoprecipitation.

**Gelatin Zymography, Western Blotting, and Immunoprecipitation.** Gelatin zymography, Western blotting, and immunoprecipitation were performed as described previously (22, 23). Concentrations of N-Tes-MMP, testican-2-MMP, and N-Tes, respectively), and the culture supernatants were analyzed as above.

**Wound-induced Migration Assays.** 293T cells transfected with expression plasmid for N-Tes-FLAG, N-Tes–Δ112-FLAG, and/or testican 2-1A were cultured on 12-well plates coated with collagen for 24 h. After removing cells by washing with 0.025% EDTA, U251 cells stably expressing MT1-MMP (2 × 10⁵ cells/well) in DMEM containing 10% fetal bovine serum were plated.

**Clinical Samples and Histology.** The classification of human brain tumors used in this study is based on the revised WHO criteria for tumors of the central nervous system (24). Fifty-one astrocytic tumors consisted of 9 low-grade astrocytomas, 8 anaplastic astrocytomas, and 34 glioblastomas. All of the tumor tissues were obtained at primary resection, and none of the patients had undergone any therapeutic intervention before resection.

**Real-Time Quantitative RT-PCR.** Real-time quantitative RT-PCR was performed as described previously with primers listed in Table 1. The mRNA level of each testican gene was expressed as a molar ratio to GAPDH mRNA level.

**ISH.** Specific antisense oligonucleotide DNA probes for ISH were designed complementary to the mRNA transcripts (Table 2). The specificity of each probe was confirmed as described previously (25). All of the DNA probes were synthesized with six biotin molecules (hyper biotinylated) at the 3′ end via direct coupling using standard phosphoramidite chemistry (Research Genetics, Huntsville, AL).

**Statistics.** Statistical analyses were performed using the χ² test and the two-tailed Mann-Whitney U test. P < 0.05 were considered significant.
by both MT1-MMP and MT3-MMP were inhibited by testican 1, 3, and N-Tes as was observed with TIMP-2. Coexpression of testican 2 abolished the inhibition by testican 1, 3, and N-Tes, whereas inhibition by TIMP-2 was not affected. Pro-MMP-2 activation mediated by MT3-MMP was inhibited by N-Tes, which was abrogated by the expression of testican 2 dependent on the dose of testican 2 plasmid (Fig. 1B). The concentrations of N-Tes in culture supernatants ranged from 840 to 950 ng/ml, and the highest testican 2 concentration in the supernatants was ~920 ng/ml.

**Mapping of Domains for the Interaction between Testican 2 and N-Tes.** To identify the domain of N-Tes responsible for the interaction with testican 2, expression plasmids for N-Tes deletion mutants were constructed (Fig. 2, A and B). Testican 2-Δ89 and testican 2-Δ133 were both detected as two bands, one of which may represent monomer and the other dimer form for each protein as estimated from molecular size. Expression of a deletion mutant of N-Tes, which consists of NH$_2$-terminal 122-amino acid residues (N-Tes-Δ122), inhibited pro-MMP-2 processing mediated by MT1-MMP or MT3-MMP as was observed with N-Tes (Fig. 2C, top panel). Expression of testican 2 abolished inhibition by N-Tes; however, inhibition by N-Tes-Δ122 was not affected by testican 2. A chimeric protein between N-Tes and testican 2, which consists of a signal peptide and the unique domain of N-Tes and other domains of testican 2 (N-Tes/Tes-2) inhibits MT-MMP-mediated pro-MMP-2 activation as shown with wild-type N-Tes. However, unlike wild-type N-Tes, inhibition by N-Tes/testican 2 chimera was not restored by testican 2 (Fig. 2C, top panel). Deletion of COOH-terminal EC domain from N-Tes was enough to abrogate the susceptibility to testican 2 (data not shown), suggesting that testican 2 interacts with the COOH-terminal EC domain of N-Tes.

Next, to identify the domain of testican 2 responsible for the inactivation of N-Tes, testican 2 deletion mutants were constructed, which comprised the NH$_2$-terminal 89- and 133-amino acid residues (testican 2-Δ89 and testican 2-Δ133), respectively (Fig. 2A). These deletion mutants still abrogated inhibition of MT-MMPs by N-Tes as wild-type testican 2 did (Fig. 2C, bottom panel). These results suggest that the NH$_2$-terminal unique domain of testican 2 interacts with the EC domain of N-Tes.

**Testican 2 Forms a Complex with N-Tes.** To examine the interaction between N-Tes and testican 2, communoprecipitation of these proteins was tested (Fig. 3). N-Tes/HA and testican 2-FLAG plasmids were transfected either alone or simultaneously. Immunoprecipitation was performed with anti-FLAG antibody (left), and the precipitates were analyzed by Western blotting with anti-HA antibody (a-HA). The blot was reprobed with anti-FLAG antibody (a-FLAG). Whole cell lysates were also immunoblotted with anti-HA antibody to confirm an equal expression of N-Tes/HA (WCL). Immunoprecipitates prepared as above using anti-HA antibody (right) were also analyzed by anti-FLAG (a-FLAG) or anti-HA antibody (a-HA). Whole cell lysates were immunoblotted to confirm an equal expression of testican 2-FLAG with anti-FLAG antibody (WCL). B, cells were transfected with expression plasmids for N-Tes/HA, testican 2-FLAG, and its deletion mutants (Δ89-FLAG and Δ133-FLAG) and analyzed as above. C, cells were transfected with expression plasmids for N-Tes/HA, N-Tes-Δ122-FLAG (Δ122-FLAG), and testican-2-FLAG, and analyzed as above.
coimmunoprecipitated with N-Tes (Fig. 3A, right panel). N-Tes-HA was also coprecipitated with testican 2-FLAG deletion mutants (testican 2-/H900489-FLAG, testican 2-/H9004133-FLAG; Fig. 3B); however, N-Tes-HA deletion mutant lacking EC domain (/H9004122-HA) failed to be coprecipitated with testican-2-FLAG (Fig. 3C). These results indicate that testican 2 binds to the COOH-terminal EC domain of N-Tes through its NH₂-terminal unique domain.

**Testican 2 Abrogates Reduction of MT1-MMP-mediated Cell Migration by N-Tes.** Stable transfection of MT1-MMP in U251 cells was confirmed by gelatin zymography, in which Mr 64,000 and 62,000 gelatinolytic bands, representing the intermediate and active form of MMP-2, respectively, were observed (Fig. 4A). Migration of parental U251 cells on collagen-coated dish in serum-free medium was minimal (Fig. 4B). Expression of MT1-MMP in U251 cells induced migration on collagen, and this was completely blocked by the addition of MMP inhibitor BB-94 to the culture medium. These results indicate that migration of U251 cells on collagen under serum-free condition is dependent on MT1-MMP activity. In the presence of 10% serum in the culture medium, U251 cells migrated actively on the collagen-coated dish regardless of MT1-MMP activity (data not shown).

To examine the effect of N-Tes, N-Tes-/H9004122, and/or testican 2 on MT1-MMP-dependent cell migration, they were deposited on collagen by culturing 293T cells transfected with each gene on dishes coated with collagen. The amount of each protein deposited on collagen was analyzed by Western blotting (Fig. 4C). Both N-Tes and N-Tes-/H9004122 were deposited effectively on collagen-coated dishes. Testican 2 was deposited only at a trace level but at a moderate level when coexpressed with N-Tes. However, deposition of testican 2 was not stimulated by coexpression with N-Tes-/H9004122.

The migration of U251 cells expressing MT1-MMP on N-Tes-coated collagen was quite low compared with migration on mock-coated collagen, and coinjection of testican 2 recovered the cell migration blocked by N-Tes. The migration of U251 cells expressing MT1-MMP was also inhibited by deposition of N-Tes-/H9004122 on collagen. As described above, culturing of cells expressing both N-Tes-/H9004122 and testican 2 deposited only N-Tes-/H9004122 but not testican 2 on collagen, and thus migration of cells expressing MT1-MMP remained restricted.

**The mRNA Expression Levels of Testican Family Genes.** To evaluate the expression levels of each testican family gene, quantitative RT-PCR for each testican mRNA was performed using GAPDH mRNA as an internal standard (Fig. 5). The mRNA levels of all of the testican family genes (testican mRNA:GAPDH mRNA molar ratios) were significantly lower in glioblastoma tissues (testican 1, 2, 3, and N-Tes, 0.093 ± 0.081, 0.107 ± 0.111, 0.007 ± 0.02, and...
Fig. 6. ISH for testican family mRNA. ISH was performed as described in “Materials and Methods.” Note that strong signals for the mRNA of all testican family genes were detected in the neurons of normal brain, as indicated by arrows (A), and weak signals for testican 1 and testican 2 mRNAs but not for testican 3 and N-Tes in the neoplastic astrocytes as indicated by arrows (B). The poly(dT)$_{20}$ probe gave a strong signal in all cells, and poly(dA)$_{20}$ probe gave only a background signal in all tissue samples. Eosin counterstain. Tes-1, testican 1; Tes-2, testican 2; Tes-3, testican 3. Bar, 50 μm.

0.029 ± 0.041, respectively; n = 34) than those in normal brain tissues (0.251 ± 0.053, P < 0.01; 0.592 ± 0.224, P < 0.01; 0.182 ± 0.062, P < 0.01; and 0.359 ± 0.108, P < 0.01, respectively; n = 10), and their expression levels decrease significantly as tumor grade increases. The mean expression level of testican 2 was the highest among testican family regardless of histological grade of astrocytic tumors.

ISH. Cells expressing mRNA for testican family members in the normal brain and glioblastomas were identified by ISH (Fig. 6). The polyT probe gave a strong signal in all of the cells, but polyadenylic acid probe gave only a background signal in the normal brain and glioblastoma tissues. The signals for each testican mRNA were detected with antisense RNA probes mainly in the neurons, but also in some endothelial cells in normal brain (Fig. 6A). Only testican 1 and testican 2 mRNA was just detected in astrocytic tumor tissues, but testican 3 and N-Tes mRNA was at negligible level (Fig. 6B). These results are consistent with that of RT-PCR analysis.

DISCUSSION

We identified previously N-Tes as an inhibitor of MT-MMPs, which is encoded by a spliced variant of testican 3 gene, and showed that testican 1 and testican 3 but not testican 2 also have an inhibitory function (20). Testican 2 was identified through the screening of a human cDNA library using an expressed sequence tag related to BM-40/osteonectin/SPARC, and was shown to be expressed mainly in brain (17). Our ISH study indicated that testican 2 was localized mainly to neurons and some endothelial cells in normal brain as reported previously (17, 19). Other testican family genes, including testican 3 and N-Tes, were also coexpressed predominantly in neurons. Because BM-40 is an ECM molecule well known as a modulator of cell adhesion and proliferation (17, 26–29), testican 2 is also thought to function in tissue remodeling. Hartmann and Maurer (30) showed in vitro that testican 2 inhibits neurite outgrowth, suggesting that testican-2 may be involved in axonal pathfinding during the development of the nervous system. However, the precise function of testican 2 as a component of ECM still remained to be explored.

The present study demonstrated that testican 2 interacts with other testican family proteins and abolishes their inactivation of MT-MMPs. Testican 2 was shown to form a complex with N-Tes/testican 3 through the NH$_2$-terminal unique domain of testican 2 and EC domain of N-Tes/testican 3. Thus, N-Tes-D122 lacking the EC domain did not interact with testican 2, but it did inhibit MT-MMPs. It was rather surprising to find that testican 2 abrogates inhibition of MT-MMP-mediated pro-MMP-2 activation by N-Tes by binding to its EC domain, because this is not involved in the inhibition of MT-MMPs. The precise mechanism of interaction between testican 2 and other testican family proteins is not clear thus far, but binding of testican 2 to the EC domain of N-Tes/testican 3 may cause conformational change, which may hamper access to MT-MMPs or may sterically hinder access to MT-MMPs.

Migration of U251 cells on dishes coated with collagen under serum-free condition was greatly enhanced by MT1-MMP activity. MT1-MMP-dependent cell migration on collagen was observed only under serum-free condition, and the cells migrated more aggressively in the presence of serum independent of MT1-MMP activity. Detachment of U251 cells from collagen would be the rate-limiting step under serum-free condition, and degradation of collagen by MT1-MMP may accelerate the migration. This would be supported by the fact that MT3-MMP, which does not degrade type I collagen, did not stimulate migration of U251 cells on collagen. Binding of testican 2 to N-Tes abrogated inhibition of MT1-MMP-dependent cell migration by N-Tes, as testican 2 abolished inhibition of MT1-MMP-mediated MMP-2 activation by N-Tes. N-Tes-D122, which is unable to form a complex with testican 2, does not induce deposition of testican 2 on collagen. Thus, testican 2 did not affect suppression of MT1-MMP-mediated MMP-2 activation or cell migration by N-Tes-D122.

The mRNA levels of all of the testican family members are lower in glioblastoma samples than those in normal brain tissues, and decrease significantly as tumor grade increases. The mRNA level of testican 2 is the highest among testican family members in normal and astrocytic tumor tissues. Furthermore, mRNAs of all of the testican family members are coexpressed mainly in neurons. Although the association of testican 2 with other ECM molecules remains to be examined, these results suggest that testican 2 could be deposited in ECM of brain in a complex form with other testican family proteins.

In conclusion, testican 2 may contribute to ECM remodeling by regulating function(s) of other testican family members, which possess MT-MMP inhibitory function. N-Tes-D122, which is incorpo-
rated into ECM and refractory to inactivation by testican 2, may have potential novel function as a barrier of glioma invasion.

ACKNOWLEDGMENTS

We thank Erik Thompson for critical reading of the manuscript.

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Testican 2 Abrogates Inhibition of Membrane-type Matrix Metalloproteinases by Other Testican Family Proteins

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