Cleavage of Lumican by Membrane-Type Matrix Metalloproteinase-1 Abrogates This Proteoglycan-Mediated Suppression of Tumor Cell Colony Formation in Soft Agar

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ABSTRACT

The small leucine-rich proteoglycan lumican was identified from a human placenta cDNA library by the expression cloning method as a gene product that interacts with membrane-type matrix metalloproteinase-1 (MT1-MMP). Coexpression of MT1-MMP with lumican in HEK293T cells reduced the concentration of lumican secreted into culture medium, and this reduction was abolished by addition of the MMP inhibitor BB94. Lumican protein from bovine cornea and recombinant lumican core protein fused to glutathione S-transferase was shown to be cleaved at multiple sites by recombinant MT1-MMP. Transient expression of lumican in HEK293 cells induced expression of tumor suppressor gene product p21/Waf-1, which was abrogated by the coexpression of MT1-MMP concomitant with a reduction in lumican concentration in culture medium. Stable expression of lumican in HeLa cells induced expression of p21 and reduction of colony formation in soft agar, which were both abolished by the expression of MT1-MMP. HT1080 fibrosarcoma cells stably transfected with the human lumican cDNA (HT1080/Lum), which express endogenous MT1-MMP, secreted moderate levels of lumican; however, treatment of HT1080/Lum cells with BB94 resulted in accumulation of lumican in culture medium. The expression levels of p21 in HT1080/Lum were proportional to the concentration of secreted lumican and showed reverse corelation with colony formation in soft agar. These results suggest that MT1-MMP abrogates lumican-mediated suppression of tumor cell colony formation in soft agar by degrading this proteoglycan, which down-regulates it through the induction of p21.

INTRODUCTION

Lumican belongs to the family of small leucine-rich proteoglycans that includes decorin, biglycan, fibromodulin, keratocan, epiphycan, and osteoglycin. Lumican is the major keratan sulfate proteoglycan of the corneal stroma, but it is also expressed in other extracellular matrices, such as skin, muscle, and cartilage (1–6). Gene-targeting studies indicate that lumican plays an important role in determining the structural phenotype of the mature collagen fibril in various tissues. Decorin is also a member of the small leucine-rich proteoglycan protein family, and the phenotypes of decorin-deficient mice were very similar to those of lumican-targeted mice (7, 8). Decorin is emerging as a powerful modulator of cell growth by affecting several key elements, including matrix assembly, growth factor binding, and receptor tyrosine kinase activity (3, 9, 10). Suppression of decorin expression was found to be related to the induction of anchorage-independent growth caused by v-src in human fetal lung fibroblasts (11). Lumican was also shown to reduce colony formation in soft agar or tumorigenicity in nude mice of rat fibroblast F204 cells transformed by K-ras or v-src oncogene (12).

Matrix metalloproteinases (MMPs) are a family of Zn2+-dependent enzymes that are known to cleave extracellular matrix proteins in normal and pathological conditions (13). Currently, 23 MMP genes have been identified in humans, and they can be subgrouped into soluble-type and membrane-type MMPs (MT-MMPs; refs. 13, 14). MMPs are overexpressed in various human malignancies and have been thought to contribute to tumor invasion and metastasis by degrading extracellular matrix components (13). Thus, the level of MMP expression correlates with the invasiveness or malignancy of tumors (15, 16). Particularly, MT1-MMP, MMP-2, MMP-7, and MMP-9 have been reported to be most closely associated with tumor invasion and metastasis. Although degradation of extracellular matrix is an important aspect of MMP biology, growing evidence has shown specific processing/activation or degradation of cell surface receptors and ligands. Fas ligand (17), tumor necrosis factor α (18), the ectodomain of the fibroblast growth factor receptor-1 (19), the heparin-binding epidermal growth factor factor-20, and interleukin (IL)-8 (21) were reported to be released or activated by MMPs. MMPs also cleave and inactivate IL-1β (22), insulin-like growth factor binding proteins (23), fibrinogen and factor XII (24), the CC chemokine MCP-3 (25), CXC chemokines RANTES cell-derived factor 1 and β (26, 27), metastasis suppressor gene product KISS-1/metastin (28), and heparan sulfate proteoglycan syndecan-1 (29).

Previously, we have developed an expression cloning method to screen genes, the products of which not only modulate pro-MMP-2 activation mediated by MT1-MMP but also serve as substrates of MT1-MMP (28–31). In this study, we have identified lumican as a substrate of MT1-MMP and showed that cleavage of lumican by MT1-MMP suppressed induction of p21/Waf-1 expression by lumican, which in turn enhanced colony formation of tumor cells in soft agar.

MATERIALS AND METHODS

Proteins and Antibodies. DEME was from Sigma (St. Louis, MO). Primers were synthesized by Genset (Kyoto, Japan). A human placenta cDNA library constructed in the pEAK8 expression vector was obtained from Edge-Bio Systems (Gaithersburg, MD). Lumican protein from bovine cornea was purchased from Sigma. Recombinant MT1-MMP, the inert mutant of MT1-MMP (MT1-E/A), and MT3-MMP catalytic domains tagged with the FLAG epitope at the COOH-terminus and purified MMP-2 protein were prepared as described previously (28, 32, 33). Monoclonal antibodies against FLAG epitope and glutathione S-transferase (GST) were purchased from Sigma and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Recombinant TIMP-1 and TIMP-2 proteins and monoclonal antibody against MT1-MMP (113-B7) were gifts from Daichi Fine Chemical Co. Ltd. (Toyama, Japan).

Cell Culture. Human embryonic kidney HEK293, HEK293T, HeLa, and fibrosarcoma HT1080 cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DEME supplemented with 5% FCS.

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Expression Cloning. Expression cloning to identify genes, the products of which interact with MMP-2, MMP-9, or MT1-MMP, was done as described previously (30).

Construction of an Expression Plasmid for Lumican Tagged with FLAG. An expression plasmid for lumican tagged with the FLAG epitope at the COOH-terminus was constructed to detect lumican protein by anti-FLAG M2 antibody (Sigma). A lumican cDNA fragment, which has a BglII restriction site in place of the stop codon, was generated by PCR with lumican cDNA cloned in pEAK8 plasmid as a template and pEAK8 forward primer (TTCATTTCTCAAGCCTCAGACAGTGG)/flanking reverse primer with an extra BglII site (underlined) starting at nucleotide 1098 of lumican gene (GenBank accession no. U21128; AGAGATCTATTAAGAGTGACTTCGTTAGCAA). The

Expression of MT1-MMP was examined by Western blotting of cell lysates using anti-MT1-MMP antibody (bottom panel). B. The expression plasmid for lumican tagged with FLAG epitope (0.4 μg) or empty pSG5 plasmid was cotransfected with 1.6 μg of control plasmid (Lane –) or expression plasmid for MT-MMP family members, as indicated by number into 293T cells cultured in 35-mm diameter dishes. Culture supernatants and cell lysates were analyzed as described above (top and middle panels, respectively). MT-MMPs, except for MT2-MMP, were tagged with FLAG epitope and were detected in cell lysates with anti-FLAG M2 antibody together with lumican core protein (middle panel). MT2-MMP was detected with anti-MT2-MMP monoclonal antibody 162-4E3, which cross-reacted with MT3-MMP (bottom panel).

Fig. 1. Degradation of lumican by MT-MMPs. A. The expression plasmid for lumican tagged with FLAG epitope (Lum-FLAG) or empty pSG5 plasmid (–) was cotransfected (0.4 μg) with the indicated amount of MT1-MMP or E/A mutant plasmid (1.6 μg) into 293T cells cultured in 35-mm diameter dishes. Total plasmid DNA was adjusted to 2 μg/dish with pSG5 plasmid. At 36 hours after transfection, the culture medium was replaced with serum-free medium with or without 0.1 μmol/L BB94, and cells were incubated additionally for 24 hours. Culture supernatants and cell lysates were then analyzed by Western blotting with anti-FLAG M2 antibody as described under Materials and Methods (top and middle panels). Expression of MT1-MMP was examined by Western blotting of cell lysates using anti-MT1-MMP antibody (bottom panel). B. The expression plasmid for lumican tagged with FLAG epitope (0.4 μg) or empty pSG5 plasmid was cotransfected with 1.6 μg of control plasmid (Lane –) or expression plasmid for MT-MMP family members, as indicated by number into 293T cells cultured in 35-mm diameter dishes. Culture supernatants and cell lysates were analyzed as described above (top and middle panels, respectively). MT-MMPs, except for MT2-MMP, were tagged with FLAG epitope and were detected in cell lysates with anti-FLAG M2 antibody together with lumican core protein (middle panel). MT2-MMP was detected with anti-MT2-MMP monoclonal antibody 162-4E3, which cross-reacted with MT3-MMP (bottom panel).

Fig. 2. Cleavage of lumican protein by MT1-MMP. A. Lumican protein (6 μg) was incubated with or without recombinant MT1-MMP catalytic domain (0.6 μg) in 30 μL TNC buffer containing 0.3% Brij at 37°C for 3 hours, separated on 10% SDS-PAGE, and stained with Coomassie Brilliant Blue. B. Lumican-GST fusion protein (3 μg) was incubated with the indicated amount of recombinant MT1-MMP catalytic domain in the presence or absence of 0.1 μmol/L BB-94 as described above, separated on 10% SDS-PAGE, and analyzed by Western blotting with anti-GST antibody. C. Lumican-GST protein incubated with MMP-2 was analyzed as described above. D. Schematic representation of lumican protein and MT1-MMP cleavage sites are shown.
amplified fragment was digested with EcoRI and BgIII and was inserted into the EcoRI and BgIII sites of pSG-FLAG vector as described previously (30).

**Degradation of Lumican by MT1-MMPs.** Suppression of MT1-MMP-mediated MMP-2 processing by the expression of lumican suggested a possible interaction between MT1-MMP and lumican. To analyze the interaction between MT1-MMP and lumican, lumican tagged with FLAG epitope at the COOH-terminus was coexpressed with MT1-MMP in 293T cells, and the secreted and cell-associated lumican was examined by Western blotting (Fig. 1A). Lumican secreted into culture medium from 293T cells transfected with the lumican cDNA was detected as a broad band of about 63 kDa, whereas cell-associated lumican core protein was detected as an 40 kDa band. Coexpression of MT1-MMP reduced the concentrations of

**Screening of Human Placenta cDNA Library.** Aliquots of placental cDNA library were cotransfected with MMP-2, MMP-9 and MT1-MMP cDNA into 293T cells, and cell lysates were analyzed by gelatin zymography. Transfection with MMP-2, MMP-9 and MT1-MMP cDNA into 293T cells, and cell lysates were analyzed by Western blotting with anti-FLAG, anti-MT1-MMP, or anti-p21 antibody as described under Materials and Methods. Cell lysates were analyzed by Western blotting with anti-FLAG antibody conjugated with Alexa Fluor 680. Coexpression of MT1-MMP reduced the concentrations of

**RESULTS**

**Screening of Human Placenta cDNA Library.** Aliquots of placental cDNA library was cotransfected with MMP-2, MMP-9 and MT1-MMP cDNA into 293T cells, and cell lysates were analyzed by gelatin zymography. Transfection with MMP-2, MMP-9 and MT1-MMP cDNA into 293T cells generated a 92 kDa gelatinolytic band of latent pro-MMP-9, 68 kDa band of latent pro-MMP-2 and 64 kDa band of MMP-2 intermediate form. Transfection of one pool of cDNA partially suppressed processing of MMP-2 to the 64 kDa intermediate form (data not shown). Five cDNA clones of 30 clones isolated from this cDNA pool by a second screening suppressed MMP-2 processing to the intermediate form when cotransfected with MMP-2, MMP-9 and MT1-MMP. The size of all 5 active cDNA fragments was 1.7 kb, and the nucleotide sequence of the cDNA fragment was determined. Homology search analysis revealed that this cDNA encodes the small leucine-rich proteoglycan lumican (GenBank accession no. U21128).

**Screening of Human Placenta cDNA Library.** Aliquots of placental cDNA library was cotransfected with MMP-2, MMP-9 and MT1-MMP cDNA into 293T cells, and cell lysates were analyzed by gelatin zymography. Transfection with MMP-2, MMP-9 and MT1-MMP cDNA into 293T cells generated a 92 kDa gelatinolytic band of latent pro-MMP-9, 68 kDa band of latent pro-MMP-2 and 64 kDa band of MMP-2 intermediate form. Transfection of one pool of cDNA partially suppressed processing of MMP-2 to the 64 kDa intermediate form (data not shown). Five cDNA clones of 30 clones isolated from this cDNA pool by a second screening suppressed MMP-2 processing to the intermediate form when cotransfected with MMP-2, MMP-9 and MT1-MMP. The size of all 5 active cDNA fragments was 1.7 kb, and the nucleotide sequence of the cDNA fragment was determined. Homology search analysis revealed that this cDNA encodes the small leucine-rich proteoglycan lumican (GenBank accession no. U21128).
lumican present in the culture medium depending on the levels of coexpressed MT1-MMP, however, cell-associated lumican core protein levels were not altered by MT1-MMP expression. Treatment of cells with the MMP inhibitor BB94 blocked the MT1-MMP-mediated reduction of lumican in the culture medium. Expression of defective MT1-MMP (MT1-E/A) failed to reduce the level of extracellular lumican. Next, other members of the MT-MMP family were compared for their capacity to cleave lumican (Fig. 1B). Among the 6 members, only MT1-MMP and MT3-MMP reduced concentration of lumican secreted into culture medium. To confirm the cleavage of native lumican by MT-MMP, lumican protein purified from bovine cornea was incubated with recombinant MT1-MMP protein, and was analyzed on SDS-PAGE (Fig. 2A). Lumican protein detected as a smear band ranging from M$_r$ 85,000 to M$_r$ 120,000 disappeared after incubation with recombinant MT1-MMP. To identify the sites of lumican protein cleavage by MT1-MMP, recombinant lumican core protein fused to GST was incubated with GST and was analyzed by Western blotting with anti-GST antibody (Fig. 2B). Digestion of lumican-GST with MT1-MMP resulted in 4 major fragments, the NH$_2$-terminal sequence of which showed the cleavage of Y$_{70}$-L$_{71}$, K$_{84}$-A$_{85}$, N$_{275}$-L$_{276}$, and Q$_{285}$-L$_{286}$ peptide bonds of lumican, respectively. Digestion of lumican-GST with MT3-MMP generated similar fragments (data not shown). MMP-2 also digested lumican-GST with comparable efficiency generating similar but not identical fragments as MT1-MMP (Fig. 2C).

**Induction of p21/Waf-1 by Lumican Is Abolished by MT1-MMP.** Decorin, another member of small leucine-rich proteoglycan family, is known to induce an inhibitor of cyclin-dependent kinases, p21/Waf-1 (34–36), and thus effect of lumican on p21 expression was examined in HEK293 cells (Fig. 3A). The endogenous level of p21 was low in 293 cells, and transient expression of lumican induced p21 expression. Coexpression of MT1-MMP, which reduced the secretion of lumican, down-regulated p21 expression; however, MT1-MMP defective mutant (E/A) had no effect on either lumican or p21 expression. Treatment of cells expressing both lumican and MT1-MMP with BB94 recovered expression of lumican and p21.

Recombinant lumican-GST fusion protein was incubated with HT1080 fibrosarcoma cells, which express high levels of endogenous MT1-MMP (37), and induction of p21 was examined (Fig. 3B). The concentration of lumican-GST protein in culture medium was substantially reduced by the incubation with HT1080 cells, and simultaneous addition of BB94 recovered lumican-GST concentration. The levels of p21 induction were proportional to the concentration of lumican-GST protein in culture medium.

**Lumican Reduces Colony Formation in Soft Agar.** HeLa cells were stably transfected with either lumican, MT1-MMP alone, or cotransfected with both genes, and expression of lumican and p21 and their colony formation in soft agar were examined (Fig. 4). Transfection of lumican or MT1-MMP in HeLa cells did not substantially affect cell growth under normal conditions (data not shown). The level of p21 was low in HeLa cells and was enhanced by the expression of lumican. Coexpression of MT1-MMP abolished p21 induction concomitant with reduction of lumican secretion. Treatment of these cells with BB94 recovered both lumican secretion and p21 expression. Colony formation in soft agar by HeLa cells was suppressed by the expression of lumican, which was recovered by coexpression of MT1-MMP.
MT1-MMP. The effects of lumican expression were also studied with HT1080 cells (Fig. 5). HT1080 cells stably transfected with lumican gene (HT1080/Lum) secreted a moderate level of lumican into the culture medium, which was enhanced by treatment with BB94. Transfection of lumican gene into HT1080 cells did not substantially affect cell growth either in the presence or absence of BB94. The secretion of lumican from HT1080/Lum cells was also stimulated by the addition of recombinant TIMP-2 protein but not by TIMP-1 protein. The level of cell-associated lumican core protein was not altered by BB94 treatment. Expression of p21 was slightly induced by stable transfection of lumican into HT1080 cells and was stimulated by BB94 treatment; however, p21 expression was not affected by BB94 treatment in control HT1080 cells. Colony forming ability in soft agar was compared between control HT1080 and HT1080/Lum cells. HT1080/Lum cells formed smaller colonies in soft agar than control HT1080 cells. Addition of BB94 in soft agar had no effect on colony formation of control HT1080 cells, however, it dramatically reduced colony formation of HT1080/Lum cells. Because MT1-MMP is sensitive to TIMP-2 but not to TIMP-1 (38), selective stimulation of lumican secretion from HT1080 cells by TIMP-2 suggests the involvement of MT1-MMP in degradation of lumican. To confirm the direct interaction between lumican and MT1-MMP, pull-down experiment was done. As shown in Fig. 5D, Lumican protein from HT1080/Lum cells was coprecipitated with MT1-E/A protein.

**DISCUSSION**

Previously, we have identified claudin-5, N-Tes/testican-3, KiSS-1/metastin, and syndecan-1 by the expression cloning strategy as molecules that interact with MMPs (28–31). MT1-MMP was identified as an activator of MMP-2, and later various substrates for MT1-MMP have been identified especially by recent proteomics approach (Table 1; refs. 28, 29, 39–44). In the present study, we identified that expression of the lumican gene suppressed activation of MMP-2 mediated by MT1-MMP in 293T cells and have shown that lumican serves as a substrate for MT1-MMP. This suggests that suppression of MT1-MMP-mediated MMP-2 activation by lumican may be because of competition between MMP-2 and lumican for the interaction with MT1-MMP. Decorin, another member of the small leucine-rich proteoglycan protein family, is known to influence tumor cell growth through indirect effects on the availability of growth factors from the extracellular matrix or directly through activation of the EGF receptor and induction of the p21/Waf-1, an inhibitor of cell cycle progression.

**Table 1** Substrates for MT1-MMP and cleavage sequences

| Protein                        | Cleavage sequences*                                                                 | Reference   |
|--------------------------------|-------------------------------------------------------------------------------------|-------------|
| Lumican                        | 6PGIKY | LLYRN                                                                 | This article|
|                                | 6HIDEK | AFENV                                                                 |             |
|                                | 21TVNEN | LENYY                                                               |             |
|                                | 21LEVNO | LEKFD                                                                |             |
|                                | 71PGPOG | IAQQR                                                              | 39          |
|                                | 71PGPOG | LLGAPG | ILGPL                                                        | 39          |
| Type 1 collagen α1(I) chain    | 40DCYS | DENG                  | 40          |
| Type 1 collagen α2(I) chain    | 40TXRD | LALSE                                                               | 41          |
| Laminin 5 γ2 chain             | 31CGPV | VRAKI                                                               | 42          |
| Integrin αV                    | 40EAFTR | AN | LNKLA                                                             |             |
| Transglutaminase               | 22HIDEM | LEATA                                                               | 29          |
|                                | 21GASOG | LLDLRK                                                              |             |
| KiSS 1                         | 14WSFG | LFGK                                                                | 28          |
| Amyloid precursor protein      | 57DVDL | MISEP                                                               | 43          |
| Interleukin 8                  | 32AVLPR | SAKEL                                                               | 44          |
| Secretary leukocyte protease   | 29WAVEG | SGKSF                                                              | 44          |
| inhibitor                      | 29WAVEG | SGKSF                                                              | 44          |
| Protopurin necrosis factor α   | 6RDLSL | ISP | LA | QA | VRSSS                                            | 44          |
| Connective tissue growth factor| 17PALAA | YRLEDTF                                                            | 44          |

* MT1-MMP cleavage sites are indicated by the arrow.
cyclin-dependent kinases (45–47). We also isolated decorin gene by the expression cloning strategy and have shown that expression of MT1-MMP reduced decorin secretion and abrogated decorin-mediated p21 induction and reduction of colony formation in soft agar (data not shown). Imai et al. (48) reported that MMP-2, MMP-3, and MMP-7 cleave decorin in vitro. In contrast, less is known about lumican and other small leucine-rich proteoglycans. However, in vitro and in vivo data indicate that lumican is also important in the regulation of collagen fibril assembly (6), and lumican was also shown to reduce colony formation in soft agar or tumorigenicity in nude mice of rat fibroblast F204 cells transformed by K-ras or v-src oncogene (12).

In the present study, we have demonstrated that expression of lumican in HEK293 and HeLa cells induced expression of p21, concomitant with the reduction of colony formation in soft agar. This may suggest that reduction of colony formation in soft agar by the expression of lumican would be because of induction of p21 by lumican. Coexpression of MT1-MMP with lumican reduced accumulation of lumican and induction of p21 and restored colony formation in soft agar. HT1080/Lum cells, which express endogenous MT1-MMP, secrete moderate levels of lumican into culture medium, and treatment of cells with BB94 induced additional accumulation of lumican protein. Because MT1-MMP is selectively inhibited by TIMP-2 but not by TIMP-1 (38), selective induction of lumican deposition by TIMP-2 but not by TIMP-1 suggests that MT1-MMP reduces secretion of lumican by HT1080/Lum cells. Direct interaction between lumican protein in HT1080/Lum culture supernatant and MT1-MMP was shown by pull-down experiment. Induction of p21 in HT1080 cells was also achieved by the addition of recombinant lumican core protein into culture medium, as was reported with decorin core protein (45). Simultaneous addition of BB94 with recombinant lumican core protein inhibited degradation of lumican protein, resulting in additional p21 induction. Thus, MT1-MMP, which has been associated with invasion and metastasis by degrading extracellular matrices, may also contribute to elevated tumorigenicity of tumor cells. The mechanism of p21 induction by lumican, which might be similar to that by decorin, still remains to be elucidated.

Lumican is the most abundant proteoglycan among small leucine-rich proteoglycan family members in breast cancer (49). Although lumican mRNA expression was high within the tumor and tumor margin, lumican protein was not immunolocalized within the tumor but within the collagenous stroma. Because MT1-MMP is predominantly found within the tumor in breast cancer (16), lumican protein expressed by tumor cells would be degraded by MT1-MMP. This may account for the observation that differences between lumican levels in normal and malignant breast tissues observed by both immunohistoch- emistry and Western blot are not as marked as those seen at the level of mRNA expression (49). Decorin and probably lumican are expected to be used for gene therapy to cause retardation of tumor growth (50), and simultaneous treatment of tumors with MMP inhibitor should be considered for small leucine-rich proteoglycan gene therapy.

In summary, we have shown that degradation of lumican by MT1-MMP abrogates p21-mediated suppression of tumorigenicity by lumican. Additional studies are needed to investigate whether degradation of lumican by MT1-MMP may contribute to early tumor development and progression in human cancer.

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Cleavage of Lumican by Membrane-Type Matrix Metalloproteinase-1 Abrogates This Proteoglycan-Mediated Suppression of Tumor Cell Colony Formation in Soft Agar

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