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A role of membrane type-1 matrix metalloproteinase in the regulation of vascular smooth muscle cell proliferation in vitro

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Abstract The purpose of this study was to assay changes in membrane type-1 matrix metalloproteinase (MT1-MMP) in vivo after vascular injury and to determine the influence of antisense MT1-MMP oligonucleotide on vascular smooth muscle cell proliferation and migration in vitro. mRNA expression of MT1-MMP was investigated in balloon-injured rat common carotid artery. The effect of antisense MT1-MMP oligonucleotide on activated matrix metalloproteinase-2 productions in cultured SMCs was evaluated by zymography. The influence of MT1-MMP antisense oligonucleotide on the proliferation and migration of cultured SMCs was measured by 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrasodium bromide assay and a 24-well transwell cell culture chamber method. The expression of MT1-MMP mRNA was increased in injured arteries, reaching a peak increase at 4 days after injury. The activation of matrix metalloproteinase-2 by SMCs was inhibited slightly by antisense MT1-MMP oligonucleotide. Antisense MT1-MMP oligonucleotide significantly inhibited SMC proliferation ($p < 0.05$) but had little effect on the migration of cultured SMCs. Expression of MT1-MMP mRNA increases following arterial injury. Antisense MT1-MMP oligonucleotide regulates SMC proliferation and may therefore constitute a feasible treatment for inhibition of intimal hyperplasia.

Introduction

Vascular reconstructions and bypass grafts fail in 30 to 50% of cases due to restenosis or intimal hyperplasia.[1-4] Smooth muscle cell (SMC) proliferation and migration and extracellular matrix deposition are implicated in these pathological conditions.[5-7] Extracellular matrix degradation occurs by the activity of matrix metalloproteinases (MMPs).[8] Arterial injury induces MMP expression such as MMP-2 (72 kD type IV collagenase/gelatinase A) and MMP-9 (92 kD type IV collagenase/gelatinase B).[9-12] Previous studies have shown that MMP-2 and -9 degrade vessel wall collagens (types III, IV, and V), elastin, and vitronectin and stimulate SMC migration from the media to the intima.[13] Unlike activation of many other MMPs, the activation of MMP-2 is not mediated by serine proteases; rather, it is regulated by a cell surface activator of membranetype-1 metalloproteinases, the prototype being MT 1-MMP.[14] Expression of MT1-MMP is the

primary trigger for activation of MMP-2. MT1-MMP also degrades type I, II, and III collagens.[15] Therefore, blocking the expression of MT1-MMP may offer a new therapeutic approach to restenosis after angioplasty.

Recent studies have focused on the use of sequence-specific antisense strands to inhibit cell function. Phosphorothioate oligonucleotides targeted against various genes involved in SMC proliferation and migration have been used to inhibit restenosis.[16-19] In the present study, we attempted to block proliferation and migration of SMCs using antisense MT 1-MMP oligonucleotide, which inhibits the production of MT1-MMP and thereby may reduce activation of MMP-2.

Method

Rat common carotid artery balloon injury model

Three-month-old male Sprague-Dawley rats were used in the experiments. All animal experiments were conducted according to the Guidelines for Animal Experimentation of Yamaguchi University School of Medicine. Rats were bred in a standard laboratory and allowed free access to food and water in a temperature-controlled environment and under a 12-hour light-dark cycle.

Animals were anesthetized by intra-abdominal administration of 50 mg/kg pentobarbital. Endothelial denudation of the left common carotid artery was performed by passage of a 2F balloon embolectomy catheter (Fogarty, Irvine, CA, USA), as described previously.[20] Before this procedure and 1, 4, 8, and 14 days after injury (n=10 for each time point), fifty animals were sacrificed, and the carotid arteries were flushed with phosphate-buffered saline solution, removed, stripped of the surrounding tissue including adventitia, frozen in liquid nitrogen, and stored at -80℃.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

RNA was isolated from vessel samples using the EZNA total RNA kit (Omega, Doraville, GA, USA) according to the manufacture's instructions.

RT-PCR was performed using a RNA PCR Kit Ver.2.1 (Takara, Osaka, Japan). A total of 0.5 μg RNA was used for hybridizing first-strand cDNA, and the cDNA probe was amplified by TaKaRa Taq DNA polymerase dissolved in PCR buffer in a 50 μl reaction containing 0.2 mM α-^{32}p-labelled dNTP and 0.2 μM MT 1-MMP primer. The primer sequence was based on the published sequence for rat MT1-MMP (forward, 5'-CCCTATGCCTA-CATCCGTGA-3'; reverse, 5'-TCCATCCAT-CACTTGGTTAT-3'); and the predicted length of the PCR product was 550 base pairs (bp). The PCR protocol was 2 minute-denaturing at 94℃, followed by 29 1-minute cycles of denaturing at 94℃, 1-minute annealing at 60℃, 2 minute-extension at 72℃, and a final extension of 5 minutes. The optimal numbers of cycles for MT1-MMP and GAPDH were determined to be 29 and 22, respectively. The PCR product (25 μl) was run on a 2% agarose gel, and radioactivity was measured with a bio imaging analyzer (Fujifilm, Tokyo, Japan).

Isolation and culture of rat SMCs

Rat aortic SMCs were isolated by an explant method and cultured in dulbecco's modified eagle medium (DMEM) (Gibco, Grand island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco). Cells from outgrowths were passaged twice at a 1:4 split. SMCs, between passages 5 and 12, were harvested and used for the following studies.

Uptake of antisense by SMCs

SMCs were seeded onto a 16-well slide chamber at 5,000 cells/well. The cells were allowed to adhere and to recover for 24 hours. The supernatant was then replaced with 100 μl fresh DMEM containing 2 μM fluorescein-labeled random oligonucleotide (Biognostik, Gottingen, Gemany), and incubated for 4, 8, or 20 hours. After incubation, the supernatant was decanted, and the wells were washed twice with serum-free DMEM followed by 100% alcohol for one minute to fix and dehydrate the cells, and a non-quenching immersion oil to allow

for visualization of oligonucleotide uptake.

Zymography

Enzymatic activity of MMP-2 was evaluated by zymography analysis as described previously.[11] SMCs were cultured in a 6-well culture plate to 60-70% confluence. The medium was then removed and replaced with serum-free DMEM for 12 hours incubation. The cells were washed twice with serum-free DMEM, and medium was replaced with 1 ml fresh DMEM with or without 2 μM oligonucleotide for 20 hours to allow uptake. Then, 10 ng/ml platelet-derived growth factor (PDGF-BB) (Sigma, Saint Louis, MO, USA) was added and the cells were cultured for 72 hours. Aliquots of the medium from cultured SMCs were mixed with sample buffer, and loaded on to a 10% polyacrylamide gel containing 0.2% sodium dodecyl sulfate (SDS) and 1 mg/ml gelatin for electrophoresis at 4℃, 20 mA constant current under nonreducing conditions. After electrophoresis, the gel was washed in 2.5% Triton X-100 for 30 minutes to remove SDS. The gel was then incubated in 50 mM Tris buffer, pH 7.8, containing 10 mM $CaCl_2$ for 18 hours. The gel was stained with 0.25% Coomassie brilliant blue (Sigma).

SMCs proliferation assay

SMCs were seeded in 20 wells onto a 96-well tissue culture plate at 5,000 cells/well. Cells were allowed to adhere and to recover for 24 hours culture and then replaced with serum-free DMEM for 12 hours incubation. The medium in five wells was replaced with 100 μl DMEM containing 10 ng/ml PDGF-BB and 2 μM antisense oligonucleotide, 100 μl DMEM containing 10 ng/ml PDGF-BB and 2 μM randomized oligonucleotide, 100 μl DMEM containing 10 ng/ml PDGF-BB, and 100 μl DMEM alone, respectively. After 96 hours of culture, the proliferation of SMCs was analyzed with the Cell Proliferation Kit (Chemicon, Temecula, CA, USA) according to the manufacture's instructions.

SMCs migration assay

SMCs migration assay was performed in a 24-well cell culture plate (Nunc, Rochester, NY, USA) with 5 μm pore polycarbonate filter inserts (Kurabou, Osaka, Japan). The filter inserts were coated with Matrigel (Becton Dickinson, Franklin Lakes, NJ, USA). The stock solution of Matrigel was diluted to 300 μg/ml in serum-free DMEM. An aliquot of 200 μl Matrigel (60 μg/well) was added into each filter insert and incubated for 1 hour at room temperature under a laminar-flow hood. The coated filters were rinsed twice with 0.5 ml PBS to remove unbound

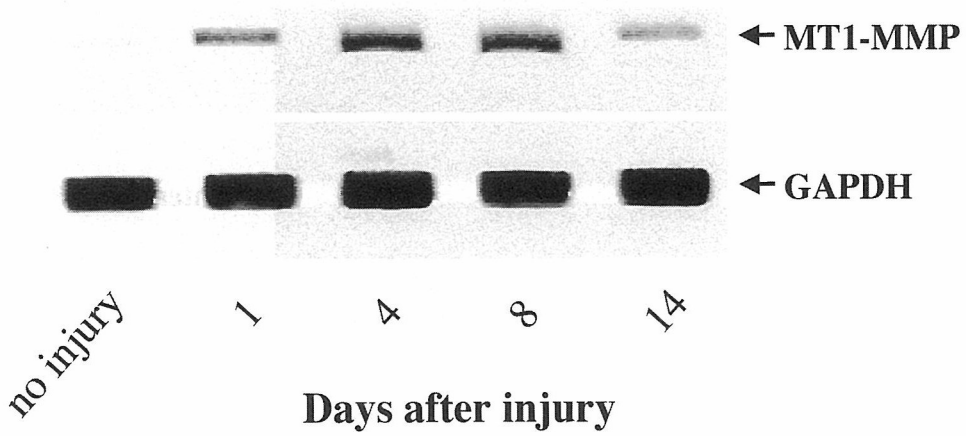

Fig. 1 mRNA expression of MT1-MMP in the injured rat carotid artery. MT1-MMP mRNA expression in arteries increased rapidly and showed a peak 4 days after balloon injury. The increased MT1-MMP mRNA expression in injured arteries was continued until 14 days.

Matrigel. SMCs (1.5 x 10^4) was seeded on the Matrigel coated filter insert and incubated for 24 hours to allow the cells to adhere and recover. The medium was then replaced with 100 μl DMEM containing 2 μM antisense oligonucleotide, 100

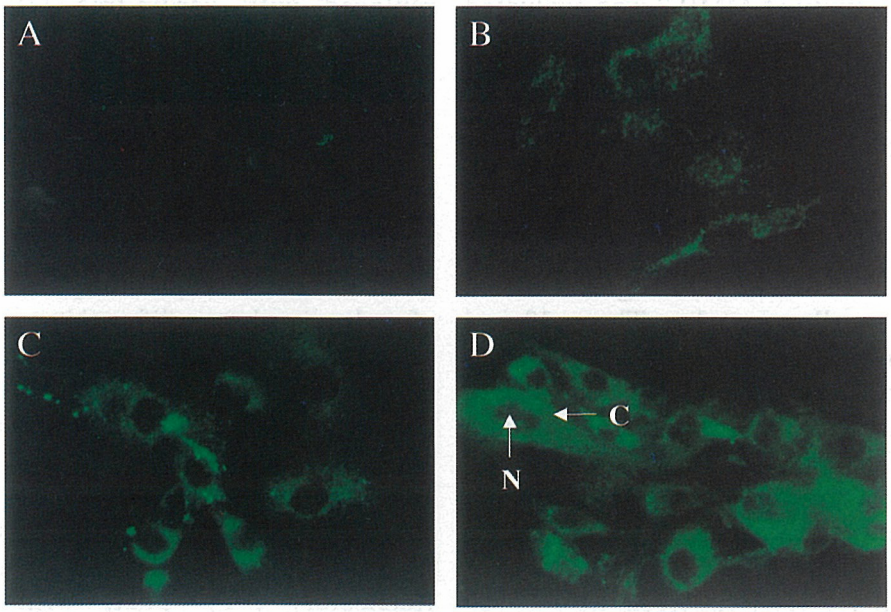

Fig. 2 Uptake of antisense MT1-MMP oligonucleotide by SMCs after 0 hour (A), 4 hours (B), 8 hours (C), and 20 hours (D) of exposure to 2 μ M FITC-labeled phosphorothioate oligonucleotide. Fluorescence in cells represented the uptake of oligonucleotide. The uptake of oligonucleotide by SMCs increased with time and peak fluorescence was identified after 20 hours (X 100, C: Cytoplasm, N: nucleus).

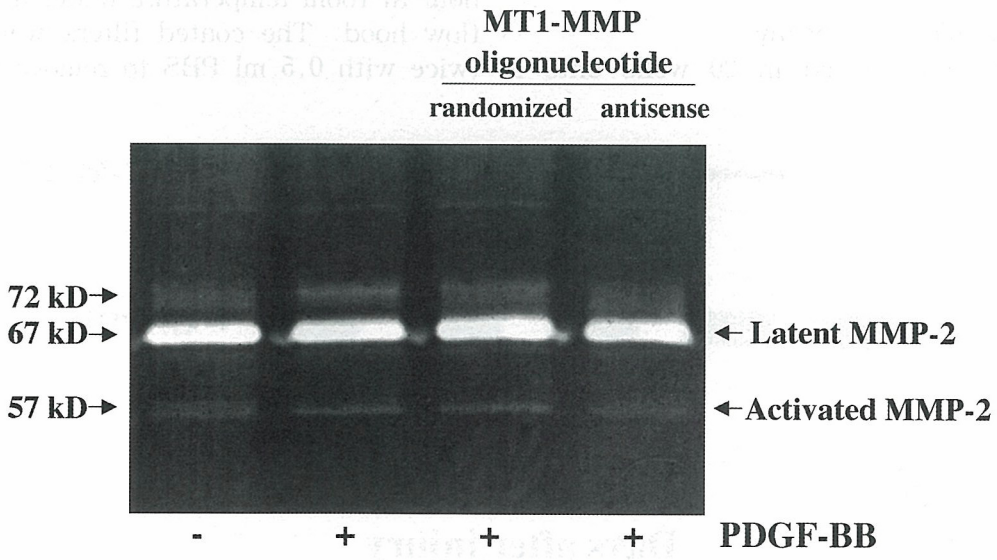

Fig. 3 Effect of antisense MT1-MMP oligonucleotide on MMP-2 activation. Dim band represented the active form of MMP-2 (57 kD) was seen in the SMCs without the addition of PDGF-BB. The active form of MMP-2 was increased in response to the addition of PDGF-BB and was inhibited mildly by the addition of antisense MT1-MMP oligonucleotide.

μl DMEM containing 2 μM randomized oligonucleotide, or 100 μl DMEM alone, respectively. After 20 hours of incubation, 400 μl DMEM containing 10 ng/ml PDGF-BB was added into each well of the culture plate. After 20 hours of culture, the number of migrated SMCs was counted under microscope with 100-fold magnification by a single observer who blinded to the experimental protocol. Migration activity was expressed as the mean number of migrated cells per 100 X field.

Statistical analysis

Results are expressed as mean±SD. Statistical analysis was performed by ANOVA and unpaired t-test with a p-value of < 0.05 considered significant.

Results

MT1-MMP mRNA expression in injured arteries

The expression of MT1-MMP mRNA was increased rapidly in the arteries following balloon injury, and showed a peak at 4 days after injury (Fig 1). The enhanced expression of MT1-MMP mRNA did not return to normal values until 14 days after injury.

Uptake of oligonucleotide by cultured SMCs

To determine whether oligonucleotide was taken into cultured SMCs, the distribution of fluorescently-labelled random oligonucleotide in SMCs was observed under fluorescence microscopy. No fluorescence was identified in the absence of oligonucleotide (Fig. 2A). The uptake of oligonucleotide by SMCs increased with time (Fig. 2B - D), and peak fluorescence was identified after 20 hours of culture with 2 μM oligonucleotide.

Effect of antisense MT1-MMP oligonucleotide on the activation of MMP-2

The effect of antisense MT1-MMP oligonucleotide on the activation of MMP-2 was examined by gelatin zymography. As showed in Fig 3, SMCs constitutively express the latent forms of MMP-2 (72 and 67 kD) but weakly express the active

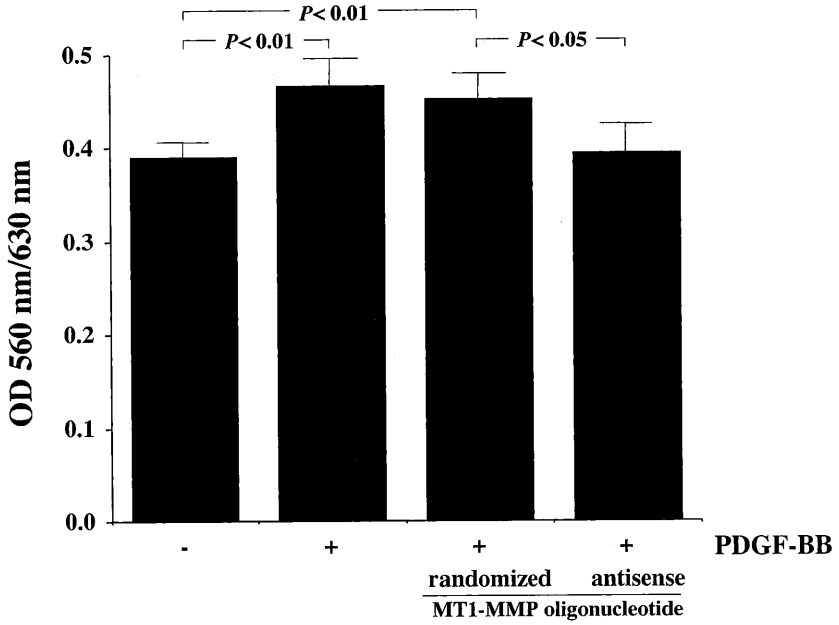

Fig. 4 Effect of antisense MT1-MMP oligonucleotide on SMCs proliferation. The optical density (OD) in each well of culture plate was measured by an enzyme-linked immunoabsorbent assay (ELISA) plate reader (Toyo, Zama, Japan) at a test wavelength of 560 nm and a reference wavelength of 630 nm. PDGF-BB stimulated the proliferation of SMCs ($p < 0.01$ vs loss of PDGF-BB). Antisense MT1-MMP oligonucleotide significantly inhibited SMCs proliferation ($p < 0.05$ vs randomized and PDGF-BB alone).

form of MMP-2. The active form of MMP-2 (57 kD) was increased in response to the addition of PDGF-BB. PDGF activation of MMP-2 was inhibited by the addition of antisense MT1-MMP oligonucleotide, but no influence was observed by the randomized oligonucleotide.

Inhibition of SMC proliferation but no effect on migration by antisense MT1-MMP oligonucleotide

The effect of oligonucleotide on SMC proliferation was measured by determining cleavage activity of MTT as a reflection of the number of living cells. As shown in Fig. 4, PDGF stimulated conspicuously the proliferation of SMCs (vs no PDGF; $p < 0.01$). The randomized oligonucleotide had no effect on SMC proliferation in comparison to the effect of PDGF alone. However, antisense oligonucleotide significantly inhibited SMC proliferation (vs randomized oligonucleotide; $p < 0.05$).

The influence of antisense MT1-MMP oligonucleotide to rat SMCs migration activity was analyzed using Matrigel coated polycarbonate filter. We observed an increase in SMC migration when 10 ng/ml PDGF-BB was added to the medium compared to that without PDGF-BB addition. Although the migrated SMCs showed about 25% less following the addition of antisense MT1-MMP oligonucleotide compared to the addition of randomized oligonucleotide, antisense MT 1-MMP oligonucleotide (2 μM) did not significantly reduce SMCs migration through Matrigel ($p = 0.10$ vs randomized) (Fig. 5).

Discussion

SMC proliferation and migration are recognized as typical responses of vascular tissues to injury, and these phenomena may contribute to undesirable sequelae.[21,22] SMCs in normal media are in a differentiated and quiescent state, and contribute to vascular contraction, whereas SMCs under pathologic conditions acquire high growth and migration abilities.[23] Intimal hyperplasia of blood vessels in response to injury is an important process in certain vascular disorders such as atherosclerosis and

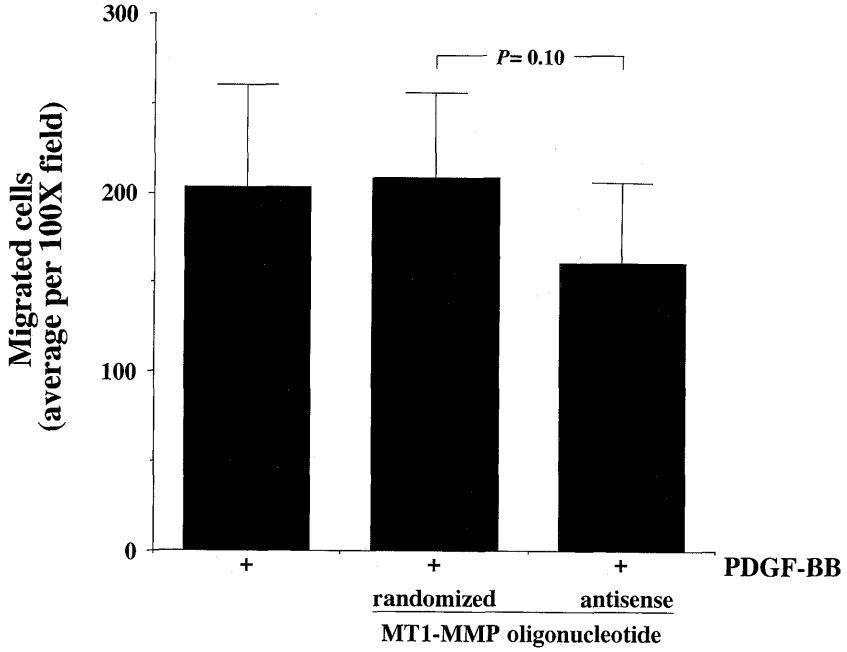

Fig. 5 Effect of antisense MT1-MMP oligonucleotide on SMC migration. SMC migration assays were performed in 24-well culture plate with 5 μm pore polycarbonate filter inserts coated with Matrigel. Although there was a trend toward inhibition of SMC migration by antisense MT1-MMP oligonucleotide, no statistically significant difference was identified ($p = 0.10$).

restenosis after angioplasty and vascular grafting. The thickened intima is composed of dedifferentiated and activated SMCs as well as extracellular matrix (ECM) proteins secreted by these activated cells.

Many studies have shown that phenotypic properties of SMCs are controlled by several ECM proteins including type IV collagen, fibronectin, and growth factors including PDGF.[24-26] When SMCs proliferate and migrate from the media to the intima, MMPs act to degrade surrounding vascular macromolecules such as type IV collagen, laminin, elastin, and proteoglycans.[11]

The zinc-dependent MMPs are thought to be involved primarily in the turnover of various ECM proteins in many physiologic and pathologic processes such as tumor cell invasion, arthritis, wound healing, embryogenesis, and angiogenesis.[27-29] MMP-2 in particular is thought to be important in tumor metastasis because of its potent degrading activity toward the basement membrane type IV collagen and its expression in many tumor types. Much attention has been paid to the role of MT 1-MMP in the activation of MMP-2 in tumor invasion and metastasis.[30-32] However, intimal hyperplasia after arterial injury does not appear to be sufficient to activate MMP-2.

In the present study, we showed minimal expression of MT1-MMP mRNA in the normal arterial wall. However, MT1-MMP mRNA expression was enhanced in injured arteries. Furthermore, the peak MT1-MMP expression was observed at 4 days after injury, it appears that MT1-MMP expression correlating to the appearance of activated MMP-2 at 5 days after injury.[11] It indicates that the enhanced expression of MT1-MMP may activate MMP-2 and increase the proliferation and migration of SMCs after injury. In fact, it has been shown that MMP-2 is specifically activated at the cell surface by MT1-MMP.

Recombinant MT1-MMP is known to digest several ECM proteins such as types I to III collagen, gelatin, fibronectin, and laminin-1. Previous studies have shown that a synthetic MMP inhibitor, BB94, inhibits SMC proliferation and migration and reduces intimal hyperplasia,[33] suggesting a therapeutic effect on restenosis after angioplasty or vascular grafting.

Antisense sequences can directly influence the expression of genes of interest and can provide a direct and unambiguous experimental approach to studying the involvement of MT1-MMP in intimal hyperplasia. In the present study, we used an antisense oligonucleotide to MT1-MMP to inhibit SMC proliferation and migration. Antisense MT1-MMP oligonucleotide inhibited the activation of MMP-2 in cultured SMCs stimulated by PDGF-BB. Our result suggests that this antisense construct has biological activity.

We analyzed the influence of antisense oligonucleotide on MT1-MMP in the proliferation and migration of cultured SMCs. We found that antisense MT1-MMP oligonucleotide significantly inhibited proliferation but had no effect on the cell migration. The inhibitory mechanism of the oligonucleotide on SMC proliferation is unclear. It is possible that the inhibition of MMP-2 activation influences the expression of proliferation-related genes and phenotypic changes of SMCs.[25] Previous studies have demonstrated that activation of MMP-2 is required as an autocrine proliferation factor for cultured hepatic stellate cells, and this proliferation is inhibited by antisense MMP-2 oligonucleotide [34] or synthetic inhibitors of MMP.[35] The inhibition of SMC proliferation in this study may have been due to the inhibition of activated MMP-2 by antisense MT1-MMP oligonucleotide. Although it has reported that the cell migration could be decreased by antisense MT1-MMP oligonucleotide,[36] we did not observe a significant influence on the migration of SMCs by antisense MT1-MMP oligonucleotide. The cells used for migration analysis in this study were not cell lines, but were harvested from rat aorta by culture. These SMCs harvested from rat aorta might be a weaker migration potential than these from cell lines. It may also be that the inhibition of MMP-2 activation by antisense MT1-MMP oligonucleotide was too weak to influence SMCs migration.

In summary, we found that the mRNA expression of MT1-MMP is increased rapidly in arteries after balloon injury. Antisense MT1-MMP oligonucleotide showed significant inhibition of proliferation but had little effect on the migration of cultured SMCs. Our results suggest that antisense MT1-MMP oligonucleotide may be an feasible treatment for atherosclerosis or restenosis after angioplasty or vascular grafting.

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