Matrix Metalloproteinase (MMP)-3 Activates MMP-9 Mediated Vascular Smooth Muscle Cell Migration and Neointima Formation in Mice

Jason L. Johnson, Amrita Dwivedi, Michelle Somerville, Sarah J. George, Andrew C. Newby

Objective—Several matrix metalloproteinases (MMPs) have been implicated in extracellular matrix destruction and other actions that lead to plaque rupture and myocardial infarction. Conversely, other MMPs have been shown to promote vascular smooth muscle cell (VSMC)–driven neointima formation, which contributes to restenosis, fibrous cap formation, and plaque stability. MMP-3 knockout reduced VSMC accumulation in mouse atherosclerotic plaques, implicating MMP-3 in neointima formation. We therefore investigated the effect of MMP-3 knockout on neointima formation after carotid ligation in vivo and VSMC migration in vitro.

Methods and Results—Twenty-eight days after left carotid ligation, MMP-3 knockout significantly reduced neointima formation (75%, P<0.01) compared with wild-type (WT) littermates, and also reduced remodeling of ligated and contralateral carotid arteries. Gelatin zymography illustrated that MMP-3 knockout abolished MMP-9 activation in ligated carotids and scratch-wounded VSMC cultures. MMP-3 knockout also attenuated VSMC migration into a scratch wound by 59% compared with WT cells. Addition of exogenous MMP-3 or activated MMP-9 restored migration of MMP-3 knockouts to that of WT VSMCs, but exogenous MMP-3 had no effect on migration in MMP-9 knockout VSMCs. MMP-9 knockout or knockdown with siRNA significantly retarded VSMC migration to the same extent as MMP-3 knockout.

Conclusion—These results indicate for the first time that MMP-3 mediated activation of MMP-9 is required for efficient neointima formation after carotid ligation in vivo and for VSMC migration in vitro, whereas MMP-12 plays a redundant role. These findings add to the understanding of MMP action in plaque stability and restenosis. (Arterioscler Thromb Vase Biol. 2011;31:00-00.)

Key Words: genetically altered mice ■ metalloproteinases ■ restenosis ■ vascular biology ■ smooth muscle cells

Accumulation of vascular smooth muscle cells (VSMCs) and their associated extracellular matrix contributes to occlusive cardiovascular pathologies, including restenosis and atherosclerosis. In the healthy blood vessel, VSMCs reside within the media in a quiescent state. However, after injury, they migrate into the intima, where their growth can result in restriction of normal blood flow. Excessive intimal thickening may compromise lumen patency directly (eg, in the case of restenosis) or accelerate the genesis of superimposed atherosclerosis (eg, in native arteries or vein grafts). On the other hand, VSMC growth within the fibrous cap of an atherosclerotic plaque is considered favorable because it is associated with a stable lesion phenotype, less susceptible to plaque rupture and its deleterious clinical sequelae.

The matrix-degrading metalloproteinases (MMPs) are a large family of genetically related enzymes that were defined initially by their ability to degrade many of the extracellular matrix components that are found within healthy and diseased blood vessels. More recently, a variety of nonmatrix substrates, including cytokines and cell surface proteins, have also been identified. As a consequence, 2 major roles in the development of cardiovascular pathologies have been attributed to MMPs: net degradation of the extracellular matrix, and regulation of the migration, proliferation, and apoptosis of vascular (including inflammatory) cells. Matrix degradation and endothelial and inflammatory cell actions clearly favor plaque rupture, but effects on VSMCs may enhance cap formation and improve plaque stability.

A large number of studies have investigated the roles that MMPs play in regulating the behavior of VSMCs both in vitro and in vivo. Liberation of VSMCs from their surrounding basement membrane and disruption of cell-cell contacts clearly permit VSMC migration into the intima; increased proliferation and apoptosis may also result. Consistent with this, nonspecific MMP inhibitors or overexpression of tissue inhibitors of MMPs retard VSMC migration and the ensuing neointima formation.
Arguably the most important studies have addressed which MMPs mediate plaque stabilizing versus destabilizing actions because such information is necessary for the development of appropriate selective inhibitors. Previous work clearly established that the gelatinases MMP-2 and MMP-9, which share the ability to degrade basement membranes, promote neointima formation in mouse models.\textsuperscript{9–12} Consistent with this, knockout (KO) of MMP-2 and MMP-9 led to decreased accumulation of VSMCs in atherosclerotic plaques of apolipoprotein E-null mice.\textsuperscript{13,14} MMP-3 KO similarly decreases accumulation of VSMCs in atherosclerotic plaques,\textsuperscript{14} which suggests that MMP-3 may also stabilize plaques by mediating VSMC migration and neointima formation. MMP-3 is a known activator of pro-MMP-9,\textsuperscript{15–17} and interestingly, MMP-3 expression and secretion may precede MMP-9 expression and activation during VSMC migration in vitro and in vivo.\textsuperscript{18} To investigate the role of MMP-3 further, we measured the effects of MMP-3 KO on neointima formation after carotid ligation in mice and on VSMC migration in culture. MMP-12 is also expressed in murine VSMCs and is elevated in neointimal lesions in both the rat carotid artery balloon injury model and the human saphenous vein organ culture model.\textsuperscript{19} The role of MMP-12 was therefore also considered.

**Methods**

**Animals and Surgical Procedure**

MMP-3, MMP-9, and MMP-12 KO mice and relevant WT littermates were bred in our laboratory and genotyped as previously described.\textsuperscript{12} Ligation of the left common carotid artery was performed as described previously.\textsuperscript{20} Carotid arteries were harvested 7 and 28 (n = 12 per group) days after surgery, fresh for biochemical assays, or after pressure-perfusion fixation using 10% formalin in phosphate-buffered saline for histological and immunohistochemical analysis.

**Histology and Immunohistochemistry**

Carotid arteries were microdissected to enable the artery to be laid flat, including removal of the suture, and preembedded horizontally using 1% (wt/vol) agar in phosphate-buffered saline. The tissue containing agar pellets were then processed for paraffin embedding. Serial longitudinal sections (3 μm in thickness) were cut through the entire width of the artery (approximately 150 μm). Multiple sections encompassing 12 μm at 24-μm intervals were subjected to hematoxylin and eosin staining and Picosirius red staining. Sections were also immunohistochemically labeled for smooth muscle α-actin (Sigma), macrophages (Mac-2, Cedarlane), apoptosis (cleaved caspase-3, R&D Systems), and proliferation (PCNA, Abcam). Additionally, immunohistochemistry for MMP-2, MMP-3, MMP-9, and MMP-12 (all Abcam) was also performed. Morphometric analysis and cell counts were performed on longitudinal arterial sections by using a computer-assisted image analysis system (ImageProPlus, MediaCybernetics).

**Gelatin Zymography**

Fresh carotid arteries were finely chopped and extracted with the use of ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 6.8, 10% glycerol, and 1% SDS) for 1 hour at 4°C, and centrifuged at 10,000g for 5 minutes. After analysis for protein content (Micro BCA assay kit, Pierce), gelatinase activity was detected by loading equal amounts of tissue extract protein (15 μg) onto 10% SDS-PAGE gels containing 1% gelatin. Bands of lysis in Coomassie Blue-stained gels were quantified using a Bio-Rad GS-690 scanning densitometer (Bio-Rad). Values were derived for bands corresponding to the pro- and activated forms of MMP-9 and expressed as optical units.

**In Vitro Studies**

VSMCs were isolated from aortic explants harvested from MMP-3 and MMP-9 KO mice and relevant WT littermates, as previously described.\textsuperscript{21} Migratory activity of both MMP-3 and MMP-9 KO and relevant WT VSMCs were compared by assessing the average closure of duplicate scratch wounds 24 hours after wounding of confluent monolayers (1×10⁵) in serum-free conditions, grown on glass coverslips. Cells were washed with sterile PBS, and 2 parallel wounds were achieved by scraping across the monolayer with a 1-mL pipette tip in a vertical position. Cells were washed once more with PBS and then incubated in serum-free Dulbecco’s modified Eagle’s medium supplemented with antibiotics, platelet-derived growth factor-BB (20 ng/mL), interleukin-1β (10 ng/mL), and hydroxyurea (2 mmol/L) to eliminate any proliferative response, for 24 hours. To assess the involvement of MMP activities on migration, replicates were additionally supplemented with either recombinant pro-MMP-3 (20 nmol/L, R&D Systems), recombinant pro-MMP-9 (20 nmol/L, R&D Systems), recombinant active MMP-3 or MMP-9 (20 nmol/L, R&D Systems, activated by incubation with 1 mmol/L APMA at 37°C for 2 hours), the nonspecific MMP inhibitor BB-94 (1 μmol/L, a MMP-9 neutralizing antibody (10 μg/mL, R&D Systems), and 1% dimethyl sulfoxide or mouse IgG (10 μg/mL, Sigma) to act as relevant controls. After 24 hours of incubation, the conditioned media were collected and subjected to gelatin zymography as described above. Cells were washed with phosphate-buffered saline and fixed in 3% paraformaldehyde, and the nuclei were stained with hematoxylin. The average area of the wound unpopulated by cells was determined using a computer-assisted image analysis system (ImageProPlus, MediaCybernetics) in 3 independent experiments.

Two silencing RNA (siRNA) oligonucleotides for MMP-9 and green fluorescent protein were purchased from Qiagen (catalogue numbers SI00206920, SI00288183). Transfections of VSMCs were performed with a Nucleofector device and VSMC kit (Amaxa, Inc., Cologne Germany) following the manufacturer’s instructions as previously described.\textsuperscript{14} Briefly 8×10⁴ cells were subjected to nucleofection with 250 pmol of MMP-9 or green fluorescent protein siRNAs using the A-33 program for mouse VSMCs. Treated cells were analyzed 24 hours after nucleofection.

Proliferation and apoptosis frequencies were determined from MMP-3 KO and relevant WT VSMCs with and without nucleofection with 250 pmol of MMP-9 or green fluorescent protein siRNAs, or, with recombinant active MMP-9 (20 nmol/L) or 1% dimethyl sulfoxide, as described previously.\textsuperscript{22} MMP-2 expression was detected by Western blotting and gelatin zymography, using pro-MMP-2 (20 nmol/L, R&D Systems) as a positive control. For in situ zymography MMP-3 WT and KO VSMCs were cultured on glass coverslips and immediately after scratch-wounding were incubated for 18 hours in the presence of fluorescently quenched DQ gelatin substrate (40 μg/mL). Parallel experiments with either recombinant MMP-9 neutralizing antibody (10 nmol/L) or mouse IgG (10 nmol/L) were performed. Gelatinolytic activity was visualized under fluorescent microscopy as previously described.\textsuperscript{23} Expression of MMP-2, MMP-3, MMP-9, and MMP-12 mRNA was assessed by quantitative polymerase chain reaction. In brief, total RNA was extracted from MMP-3 WT and KO VSMCs before and 24 hours after wounding, using RNasey Mini kit (Qiagen) according to the manufacturer’s instructions. Quantity and purity were assessed by absorbance at 260 and 280 nm. cDNA was generated using 4 U of RT enzyme (Promega) per 1 μg of RNA. The cDNA was amplified by quantitative polymerase chain reaction using a Roche Light Cycler using 55 cycles for tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3, MT1-MMP, and 18S, of 94°C for 15 seconds, 58°C for 20 seconds, 72°C for 20 seconds, and 72°C for 20 seconds). The forward and reverse primers (Sigma) were 5’-GGCTGACATCATGATCAACCTTGGG-3’ and 5’-GCCATACGCTTCCATCATTTTAC-3’ for MMP-2, 5’-GCATTCCCTGATGTCCCTGTTG-3’ and 5’-TCCCGGAGGGTCGTGACT-3’ for MMP-3, 5’-GGAACACACCAGGAACATCCACTTTAC-3’ for TIMP-1, 5’-GAACAGAGGACAGGAAGATT-3’ for TIMP-2, 5’-GACAGACATCAGCTAAGATG-3’ for TIMP-3, and 5’-GGCTGACATCATGATCAACCTTGGG-3’ and 5’-GCCATACGCTTCCATCATTTTAC-3’ for MMP-2, 5’-GCATTCCCTGATGTCCCTGTTG-3’ and 5’-TCCCGGAGGGTCGTGACT-3’ for MMP-3, 5’-GGAACACACCAGGAACATCCACTTTAC-3’ for TIMP-1, 5’-GAACAGAGGACAGGAAGATT-3’ for TIMP-2, 5’-GACAGACATCAGCTAAGATG-3’ for TIMP-3.
and 5'-AGAGAGGAGTCTGGGGTCTGGTTT-3' for MMP-9, 5'-CCTCGATGTGGAGTGCCCGA-3' and 5'-CCTCAGCTCTATGTCGAGATG-3' for MMP-12, and 5'-GCCAGGGAAGACAGG-3' and 5'-GCCGCATCATGTTCTTGCCCA-3' for 36B4. Data were analyzed using LightCycler Data Analysis software.

**Statistical Analysis**
Values are expressed as mean±standard error of the mean (SEM). For the comparison of mean values, a check was first made for similar variances; if this was passed, then a Student t test was carried out. If the variances were significantly different, then a t test with the Welch correction was used. For the comparison of more than 2 groups, an ANOVA test was used. Differences were considered statistically significant when P<0.05.

**Results**

**In Vivo Effects of MMP-3 Deficiency on Neointimal Formation in Ligated Carotid Arteries**
We observed no differences in body weights or blood vessel dimensions between the KO mice and relevant WT controls before experimental inception, in agreement with our previous observations,14 and carotid arteries before ligation showed no detectable intimal growth (all results not shown). The substantial increase in neointimal area observed 28 days after ligation in MMP-3 WT mice was significantly reduced by 86% in MMP-3 KO littermates (P<0.001; Figure 1 and the Table). Additionally, neointimal lesion length was significantly reduced (75%, P<0.01; Figure 1 and the Table) in MMP-3 KO mice compared with MMP-3 WT controls. In response to ligation of the left carotid artery, compensatory expansive remodeling (assessed by an enlargement in total vessel area) takes place in the right carotid artery to accommodate for the increase in blood flow/shear stress.24 However, total vessel area of contralateral right carotid arteries was statistically significantly reduced by 35% (P<0.05) in littermate MMP-3 KO mice compared with MMP-3 WT animals (Figure 2A to 2C), which suggests a role for MMP-3 in facilitating expansive remodeling. Moreover, we observed...
greater adventitial collagen content in right carotid arteries of MMP-3 KO mice compared with MMP-3 WT animals (Figure 2D to 2F), which is associated with reduced expansive remodeling.

Neointimal lesions from both MMP-3 KO and WT mice contained mainly smooth muscle α-actin positive cells (~93% of total cells; Figure 3). The percentage of smooth muscle cell actin–positive cells within either the neointima or media was similar in both groups (Figure 3). Small numbers of macrophages were detected in both groups within neointimal lesions and the underlying media. Hence, the prevalence of macrophages measured as the ratio between macrophages and smooth muscle cells was not altered between MMP-3 KO (0.072 ± 0.015) and WT animals (0.068 ± 0.025) (Figure 3). We additionally assessed proliferation and apoptotic frequencies in neointimal cells (28 days postligation) and observed no difference between MMP-3 KO and WT mice (Supplemental Figure I, available online at http://atvb.ahajournals.org).

Production of MMP-3 was prominent by immunohistochemistry in sections from ligated carotid arteries from WT mice (Figure 4A), but in comparison was greatly reduced in WT unoperated control arteries (Supplemental Figure IIA). MMP-3 expression was prominent in the neointima but was also detected in the media (Figure 4A). Serial sections were also labeled for MMP-9, and this showed a distribution similar to that of MMP-3 (Supplemental Figure IIA and IIB). Furthermore, a significant correlation was observed in protein expression in the neointima from arteries of different animals (Figure 4A), suggesting coordinate regulation during neointimal formation. To further investigate this possibility, we performed gelatin zymography (Figure 4B). This revealed that most of the MMP-9 was present as the pro form (92 kDa) but that a small amount was activated (82 kDa). Our studies revealed no difference in the level of pro-MMP-9 in carotid arteries 7 days after ligation in MMP-3 KO mice compared with WT controls (Figure 4B). However, a significant reduction (69%; P<0.05) in the level of active MMP-9 was observed in ligated carotid lysates from MMP-3 KO mice compared with controls (Figure 4B). A reduction in neointimal formation was also observed in MMP-9 KO compared with littermate WT mice (Table), consistent with previous publications.11,12 The reduction in neointimal areas was similar in magnitude to that seen in MMP-3 KO mice (Table). Fragmentation of the elastic lamellae is also associated with enhanced smooth muscle cell migration and neointimal and was therefore assessed in MMP-3 WT and KO ligated arteries. We demonstrate that significantly more ligated carotid arteries from MMP-3 WT mice exhibited fragmented elastic lamellae (7 of 7 mice) compared with MMP-3 KO mice (1 of 7 mice; P=0.0047 by the Fisher exact test; Supplemental Figure III). Accordingly, we examined the protein expression of the potent elastase MMP-12 in ligated arteries but observed no difference between groups (Supplemental Figure IVA). Consequently, no differences in neointimal formation were detected between MMP-12 KO and littermate WT mice (Table), in contrast to the effects on neointimal formation observed in MMP-3 and MMP-9 KO mice. MMP-2 gene deletion has also been shown to retard neointimal formation and smooth muscle cell migration9; however, MMP-2 protein expression was unchanged between MMP-3 WT and KO ligated carotid arteries (Supplemental Figure IVB).

### Table. Effect of Matrix Metalloproteinase Deficiency on Neointimal Lesion Cross-Sectional Area and Length

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lesion Area (×10^3 mm²)</th>
<th>Lesion Length (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-3 WT</td>
<td>360±60</td>
<td>671±108</td>
</tr>
<tr>
<td>MMP-3 KO</td>
<td>48±33*</td>
<td>165±63*</td>
</tr>
<tr>
<td>MMP-9 WT</td>
<td>388±111</td>
<td>888±77</td>
</tr>
<tr>
<td>MMP-9 KO</td>
<td>65±27*</td>
<td>406±120†</td>
</tr>
<tr>
<td>MMP-12 WT</td>
<td>129±41</td>
<td>518±98</td>
</tr>
<tr>
<td>MMP-12 KO</td>
<td>154±28</td>
<td>518±87</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SEM. MMP indicates matrix metalloproteinase; WT, wild-type; KO, knockout.

*P<0.01.
†P<0.05.

Figure 2. Matrix metalloproteinase-3 (MMP-3) deficiency alters contralateral vessel remodeling and collagen deposition. A to C, Effect of MMP-3 gene deletion on vessel remodeling of right contralateral carotid arteries 28 days postligation of the left carotid artery of MMP-3 WT (A) and knockout (KO) (B) mice. C, Quantification of mean total vessel area of right contralateral carotid arteries. D and E, Representative images of Picosirius red-stained right contralateral carotid arteries 28 days postligation of the left carotid artery of MMP-3 WT (D) and KO (E) mice visualized under polarized light. F, Quantification of mean adventitial collagen area of right contralateral carotid arteries. Scale bars=10 μm. *P<0.05, n=8 per group.

### In Vitro Effects of MMP-3 Deficiency on VSMC Migration

To explore the mechanisms responsible for reduced neointima formation in MMP-3 KO mice we performed in vitro experiments. To quantify the effects of MMP-3 deficiency on
VSMC migration, we used cells isolated from aortic explants in a wound healing assay, conducted in the presence of hydroxyurea to eliminate the contribution of proliferation. MMP-9 activity was not detected using gelatin zymography in the conditioned medium from uninjured cultures of mouse VSMCs (results not shown). However both pro-MMP-9 and a smaller amount of activated MMP-9 were revealed in injured cultures. The levels of pro-MMP-9 and active MMP-9 were reduced by 35% ($P<0.05$) and 57% ($P<0.05$), respectively, in MMP-3 KO compared with WT VSMCs, 24 hours after wounding (Figure 4C). Concomitantly, we found that MMP-3 KO significantly (59%, $P<0.01$) impaired migration

Figure 3. Matrix metalloproteinase-3 (MMP-3) deficiency decreases neointimal smooth muscle cell (SMC) and macrophage content. Shown is immunohistochemical labeling of MMP-3 wild-type (WT) (A and C) and MMP-3 knockout (KO) (B and D) carotid arteries 28 days after ligation, for α-smooth muscle actin (A and B) and Mac-2 (C and D; arrows indicate Mac-2 positive macrophages). Scale bar in B represents 100 μm and applies to A to D. Quantification of VSMC and macrophage number as a percentage of total cells in both the neointimal lesion and media are represented in the adjoining graphs (E to H). *$P<0.05$, n=8. Dotted lines in all panels represent the IEL (media/neointima border).
of VSMCs, expressed as the average area remaining open 24 hours after subject to a 1-mm wound (Figure 5A). The addition of exogenous MMP-3 to MMP-3 KO VSMCs restored their migratory capacity to that of MMP-3 WT VSMCs (Figure 5A). MMP-3 has been shown to activate pro-MMP-9.15 Hence, the effects of MMP-3 on migration of VSMC could be mediated indirectly through MMP-9 activation. Consistent with this, enhanced migration of MMP-3 KO VSMCs was not achieved after addition of recombinant proMMP-9 (Figure 5A). However, supplementation of APMA-activated MMP-9 restored their migratory capacity to the same level as exogenous MMP-3 and back to the level seen in MMP-3 WT cells (Figure 5A). To investigate further the relationship between MMP-3 and MMP-9, the migratory pattern of MMP-9 KO VSMCs was assessed in the presence of either recombinant MMP-3 or MMP-9. Exogenous pro-MMP-9 markedly enhanced the migration of MMP-9 KO VSMCs (Figure 5B; P<0.01), which implied that MMP-9 KO cells retained the ability to activate MMP-9. However, addition of either pro- or active MMP-3 failed to enhance migratory potential of MMP-9 KO VSMCs (Figure 5B) in contrast to effects on WT cells (Figure 5A and 5B). These findings confirmed that MMP-3 did not stimulate VSMC migration directly but acted through MMP-9. To further test this hypothesis, we used siRNA for MMP-9, which we previously demonstrated significantly reduced MMP-9 protein expression by 74% (P<0.01, Figure 6). MMP-9 siRNA retarded the migration of MMP-3 WT VSMCs (P<0.01, Figure 6) but had no effect on MMP-3 KO VSMCs. Taken together, these 3 experiments consistently demonstrate that MMP-3 and MMP-9 are required to work in concert to promote VSMC migration. Subsequently, using in situ zymography, we demonstrated that wounded VSMCs from MMP-3 KO mice exhibit reduced gelatinolytic activity (74%; P<0.05).
pared with wounded WT VSMCs (Supplemental Figure V). Moreover, addition of a MMP-9 blocking antibody to WT VSMCs reduced gelatinolytic activity (76%; $P<0.05$) to that observed in KO cells (Supplemental Figure V), confirming the need of both MMP-3 and MMP-9 for efficient gelatinolysis, a prerequisite for VSMC migration. We also examined the expression of the other major gelatinase, MMP-2. MMP-2 mRNA expression was unaffected between MMP-3 WT and KO VSMCs both before and after wounding (Supplemental Figure VI). Similarly, MMP-3, MMP-9 and MMP-12 mRNA expression was unaltered (Supplemental Figure VI). Interestingly, MMP-2 protein expression was significantly decreased in wounded MMP-3 KO VSMCs compared with WT (48%; $P<0.05$; Supplemental Figure VIII) but the abundance of active MMP-2 (65 kDa) remained unchanged in the VSMCs from the MMP-3 KO mice (Supplemental Figure VII), possibly in response to a change in VSMC migratory phenotype. These findings imply that unlike MMP-9, MMP-2 activity is not reliant on MMP-3 and therefore not responsible for the effects observed in MMP-3 KO VSMCs and ligated mice. Similar to the in vivo data, MMP-3 gene deletion had no effect on VSMC proliferation rates (Supplemental Figure VIII). Furthermore, either addition of active MMP-9 or siRNA of MMP-9 failed to alter rates of proliferation (Supplemental Figure VIII). We have also recently shown that the frequency of apoptosis is also unaffected by MMP-3 gene deletion.25

**Discussion**

The present study is the first to demonstrate a role for MMP-3 in MMP-9 activation, hence VSMC migration in vitro and neointima formation and vascular remodeling in vivo. This information provides a rationale for the observation that MMP-3 KO reduces the VSMC content of lesions in apolipoprotein E–null mice, an effect consistent with impairment of plaque stability.

Previous work has implicated gelatinolytic MMPs, specifically MMP-2 and MMP-9 in VSMC migration and neointima formation (as reviewed by8). First, VSMC outgrowth from in vitro rabbit26 and baboon27 aortic explants and vascular injury in vivo in rodents28–30 and pigs31,32 are associated with increased pro-MMP-2 and MMP-9 expression and activation. Second, MMP-specific inhibitors33,34 and tissue inhibitors of MMP overexpression 35–40 attenuate VSMC migration and neointima formation in vivo, with less consistent effects on proliferation.41 Third, deficiency of MMP-29,10 MMP-1442 and MMP-911,12 in murine vascular injury models reduces neointima formation and VSMC migration, whereas proliferation is again less affected. VSMCs from MMP-9 KO mice show reduced migration in vitro,10,12 as we confirmed here, whereas VSMCs transduced with MMP-9 have greater migratory properties both in vitro and in vivo.22 These above data define the role of several MMPs in VSMC migration and neointima formation, but the roles for other MMPs remain unclear. The processes responsible for MMP-9 activation during VSMC migration are also uncertain. In vitro studies demonstrate the capacity of the urokinase-type plasminogen activator/plasmin system to activate MMP-9.44–46 However, we found that MMP-9 activation is maintained after mouse carotid artery ligation in urokinase-type plasminogen activator KO mice (A. C.

![Image](http://atvb.ahajournals.org/)

**Figure 5.** VSMC migration is dependent on matrix metalloproteinase-3 (MMP-3) and MMP-9 activity. A, Migration assessed 24 hours after wounding in VSMCs from MMP-3 wild-type (WT) and knockout (KO) mice and in MMP-3 KO VSMCs treated with recombinant MMP-3, recombinant pro-MMP-9, or recombinant active MMP-9. *Significant difference from MMP-3 WT VSMCs ($P<0.05$); #significant difference from MMP-3 KO VSMCs ($P<0.05$). B, Migration assessed 24 hours after wounding in VSMCs from MMP-9 KO mice treated with either recombinant pro-MMP-3, recombinant active MMP-3, or recombinant pro-MMP-9 (rMMP-9). *Significant difference from untreated smooth muscle cells ($P<0.05$).

![Image](http://atvb.ahajournals.org/)

**Figure 6.** Depletion of matrix metalloproteinase-3 (MMP-3) activity modulates MMP-3 knockout (KO) VSMC migration. Migration assessed 24 hours after wounding in VSMCs from MMP-3 wild-type (WT) and KO mice treated with either MMP-9 siRNA (250 pmol) or green fluorescent protein siRNA (siGFP) as a control (250 pmol). *Significant difference from green fluorescent protein siRNA–treated MMP-3 WT VSMCs ($P<0.05$).
Newby, unpublished observations). We therefore considered the possibility that MMP-3, which can effectively activate proMMP-9 in vitro using either cancer cell lines or purified proteins,15–17 might be capable of activating MMP-9 after arterial injury.

Hultgårdh-Nilsson and colleagues47 demonstrated elevated MMP-3 expression in VSMCs cultured on basement membrane matrix components. MMP-3 and MMP-9 are also coordinately upregulated by growth factors and inflammatory cytokines in rat VSMCs.48 Moreover, MMP-3 and MMP-9 are both upregulated coincident with VSMC migration during the first week after balloon injury in several species49 and it has also been shown that MMP-3 expression is regulated during VSMC migration and invasion50 and is increased 3 days after carotid ligation in C57Bl6/J mice.9 Moreover, MMP-3 expression precedes MMP-9 expression after balloon angioplasty of the rat carotid artery.18 The above findings were consistent with the proposal that MMP-3 may facilitate VSMC migration and neointima formation through activation of MMP-9, and we therefore focused on testing this hypothesis.

The blood-flow cessation model involving ligation of the mouse left common carotid artery induces a decrease in lumen cross sectional area via both neointima formation and medial remodeling.20 With this model, we have shown for the first time that MMP-3 and MMP-9 coordinate neointima formation. Our evidence is as follows: (1) VSMC protein expression of MMP-3 and MMP-9 both localize in the media and neointima of ligated mouse carotid arteries. (2) Either MMP-3 or MMP-9 KO reduced neointima formation and VSMC migration in vitro to a similar extent. (3) MMP-3 KO reduced activation of pro-MMP-9 in vivo and in vitro. (4) Addition of exogenous MMP-3 and active MMP-9 restored migration to MMP-3 KO VSMCs. (5) Pro-MMP-9 failed to restore migration, presumably because of lack of activation in the absence of MMP-3. (6) Knockdown of MMP-9 with siRNA was as effective as MMP-3 KO at inhibiting VSMC migration. Remodeling of the mouse carotid artery in response to ligation has also been shown to depend on MMP-9.51 Our results showing that MMP-3 KO reduces remodeling after ligation are therefore also consistent with an indirect action through MMP-9.

MMP-12 is also expressed in murine VSMCs and is elevated in neointimal lesions in both the rat carotid artery balloon injury model and the human saphenous vein organ culture model.19 Moreover, previous in vitro evidence in mouse VSMCs demonstrated that MMP-12 can cleave cadherin-mediated cell-cell contacts,19 which provides a possible mechanism to increase cell migration and proliferation. However, we show here that MMP-12 deletion had no detectable effect on neointima formation in mice 28 days after carotid ligation. Hence the in vitro effects appear to be quantitatively insignificant in the carotid ligation model.

Using an apolipoprotein E–null mouse model of atherosclerosis, we have previously established that deletion of either MMP-3 or MMP-9 KO favors an unstable plaque phenotype, characterized by a reduction in VSMC numbers.14 Our present data showing that MMP-3 and MMP-9 coordinately regulate VSMC migration in vitro and neointima formation in vivo provide a rationale for their effects on VSMC numbers in atherosclerotic plaques. Furthermore, we may infer that MMP-3 and MMP-9 specific inhibitors would be effective in reducing neointima formation but would carry the risk of promoting plaque instability, although this needs to be demonstrated directly. Moreover, it would be desirable in the future to evaluate VSMC-specific knockdown or overexpression of MMP-3 in vivo to confirm the therapeutic potential of targeting this protease for restenosis and atherosclerosis respectively. In contrast to MMP-3 and MMP-9 KO, MMP-12 KO had no effect on neointima formation in this study. Previous work showed that MMP-12 KO reduces macrophage invasion in vitro and in vivo;52 it also decreases macrophage rather than VSMC numbers in atherosclerotic plaques of apolipoprotein E–null mice.14,53 Furthermore, an MMP-12 selective inhibitor inhibits macrophage invasion, abrogates atherosclerotic plaque progression, and reduces macrophage to VSMC ratio in mouse plaques, characteristics associated with plaque stabilization in man.22 The relatively insignificant effect of MMP-12 on VSMC migration in vivo demonstrated here suggests that MMP-12 inhibitors might show a favorable therapeutic profile for plaque stabilization.

In summary, the present study demonstrates that MMP-3 participates in intimal hyperplasia in a mouse carotid artery ligation model and contributes to vessel remodeling. Furthermore, KO of MMP-3 attenuates VSMC migration through reduced MMP-9 activation. These findings support the hypothesis that MMP-3 and MMP-9, by working in concert, could be beneficial with regard to plaque stabilization by promoting fibrous cap formation. Consequently, specific inhibitors for MMP-3 and MMP-9 may have therapeutic potential toward restenosis prevention but deleterious effects on atherosclerotic plaque stability. By contrast, our results further support the therapeutic potential of MMP-12 inhibition for plaque instability because they did not reveal a major effect of MMP-12 in VSMC migration during neointima formation and, by implication, fibrous cap formation.

Acknowledgments
The authors thank Dr Christopher Jackson for support in supplying the MMP-3– and MMP-9–deficient mice.

Disclosures
None.

References


23. Southgate KM, DeSimone N, Hahn-Dantona E, Sipley J, Nagase H, French DL, Quigley JP. Activation of matrix metalloproteinase-9 (MMP-9) via a...
converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. 


Matrix Metalloproteinase (MMP)-3 Activates MMP-9 Mediated Vascular Smooth Muscle Cell Migration and Neointima Formation in Mice
Jason L. Johnson, Amrita Dwivedi, Michelle Somerville, Sarah J. George and Andrew C. Newby

Arterioscler Thromb Vasc Biol. published online June 30, 2011;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2011/06/30/ATVBAHA.111.225623

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2011/06/30/ATVBAHA.111.225623.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Figure S1

A – proliferation

Figure S1: Proliferation and apoptosis frequencies in ligated carotid arteries
Quantification of immunohistochemical labelling of MMP-3 WT and KO carotid arteries 28 days after ligation for PCNA (A) and cleaved caspase-3 (B). NS indicates no significant difference. All values are expressed as mean SEM. All groups n=3.
Figure S2: MMP-3 and MMP-9 protein expression in non-ligated carotid arteries
Immunohistochemical labelling of MMP-3 WT non-ligated carotid arteries for MMP-3 (A) or MMP-9 (B). Scale bar in panel A represents 50µm and applies to all panels.
Figure S3: Elastic lamellae fragmentation in ligated carotid arteries
Representative images of MMP-3 WT and KO carotid arteries 28 days after ligation observed under fluorescence to visualise auto-fluorescent elastic lamellae. White arrows indicate areas of elastic lamellae fragmentation. Scale bar in top left panel indicates 100µm and applies to all panels.
Figure S4

A – MMP-12

B – MMP-2

Figure S4: MMP-12 and MMP-2 protein expression in ligated carotid arteries
Immunohistochemical labelling of MMP-3 WT (i) and KO (ii) carotid arteries 28 days after ligation for MMP-12 (A) and MMP-2 (B). Quantification is represented in adjoining graphs (iii) and NS represents no significant difference. Scale bar in panel i represents 100µm and applies to all panels. All values are expressed as mean ± SEM. All groups n=3.
Figure S5

**Figure S5: In situ zymography on VSMC from MMP-3 WT and KO mice**

Gelatinolytic in situ zymography of MMP-3 WT (A-C) and KO (D-F) wounded VSMC with incubation buffer alone (A and D) or plus 100µg/ml mouse IgG (B and E), or 100µg/ml MMP-9 BAb (C and F). Green fluorescence represents gelatinolytic activity. Scale bar in Panel F represents 100 µm and is applicable to all panels. Quantification is represented in adjoining graphs (G). * denotes p<0.05 versus MMP-3 WT + IgG † denotes p<0.05 versus MMP-3 WT control. All values are expressed as mean ± SEM. All groups n=3.
Figure S6: Quantitative real-time RT-PCR analysis of MMP-3 WT and KO VSMC.
Data were obtained from MMP-3 WT and KO non-wounded and wounded VSMC and are expressed as mean SEM of mRNA normalized to 36B4 and relative to the MMP-3 WT non-wounded as a control. No significant differences were observed. All groups n=3.
Figure S7: MMP-2 protein expression and gelatinase activity in MMP-3 WT and KO VSMC

A and B; Representative western blots for MMP-2 (72kDa) and GAPDH (37kDa) in wounded MMP-3 wt and KO VSMC. Quantification of optical densities normalised to GAPDH are shown in adjoining graph (C). * denotes p<0.05 versus MMP-3 WT. All values are expressed as mean ± SEM. All groups n=3.

D, Condition media from wounded MMP-3 WT and KO VSMC were used for gelatin zymography. Recombinant pro-MMP-2 was used as a positive control (72kDa).
Figure S8: Effect of MMP-9 activity on MMP-3 WT and KO VSMC proliferation
Proliferation assessed by BrDU incorporation 24 hours after wounding in VSMC from MMP-3 WT and KO mice treated with either (A) active-MMP-9 (20nmol/L) or DMSO as a control or (B) MMP-9 siRNA (250pmol) or GFP siRNA as a control (250pmol). No significant differences were observed. All groups n=3.