Furin and membrane type-1 metalloproteinase mRNA levels and activation of metalloproteinase-2 are associated with arterial remodeling

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Abstract Matrix metalloproteinase (MMP) activation is an essential feature of pathological and physiological arterial enlargement or shrinkage. Recently, furin-activated membrane type-1 MMP (MT1-MMP) was identified as the in vivo activator of MMP2 in mice. Although arterial enlargement and shrinkage are important in several pathological processes, this proprotein convertase–MT1-MMP axis has not been described during arterial remodeling. In rabbit femoral and carotid arteries, we report an increase in furin and MT1-MMP mRNA levels before and at the onset of arterial remodeling followed by an increase in activated MMP2. This reveals the presence of the proprotein convertase–MT1-MMP axis in flow-induced arterial remodeling and identifies furin as a possible target for local intervention in pathological arterial remodeling.

Key words: Furin; Metalloproteinase; Arterial remodeling

1. Introduction

Degradation and resynthesis of the extracellular matrix are essential during tissue remodeling. Matrix turnover is necessary for physiological and pathological processes to occur, such as cell migration, angiogenesis, tumor cell invasion and wound healing.

In arteries, remodeling of the arterial wall varies from arterial enlargement to shrinkage and determines the luminal narrowing after balloon angioplasty [1–3], in atherosclerosis [4,5] and during sustained blood flow changes [6].

The major extracellular component of the arterial wall is collagen. Together with other proteins, collagen forms a structural protein network, that is rigid and resistant to proteolytic digestion. The only proteinases able to cleave collagen are matrix metalloproteinases (MMPs). These prove to play an essential role in arterial remodeling since MMP inhibitors prevent arterial remodeling after sustained flow changes and balloon injury [7–9].

MMPs are mostly synthesized as inactive zymogens (proMMPs) and their activation by proteolytic cleavage is a rate-limiting step for their catalytic function. Membrane type-1 MMP (MT1-MMP) is a membrane-anchored MMP and has a pivotal function in connective tissue metabolism [10,11] and activation of proMMP2 [12].

MT1-MMP is activated after cleavage by a proprotein convertase [13,14] and activation results in a stimulation of proMMP2 cleavage generating the activated MMP2. In tissues from MT1-MMP null mice, activation of MMP2 was deficient suggesting that MT1-MMP is essential for its activation in vivo [11].

In vitro studies show that the proprotein convertase–MT1-MMP–MMP2 axis plays a major role in regulating complex arrays of proteolytic activities [15]. However, in arteries this MMP activation axis has not yet been described. MMP activation is assumed to play an important role in arterial geometrical remodeling. We therefore hypothesized that the proprotein convertase–MT1-MMP expression precedes MMP2 activation and subsequent arterial remodeling.

MMP activity is associated with influx of macrophages [16] and neointima formation [17] which may occur simultaneously with arterial restructuring. In this study, we induced arterial remodeling without neointima formation and inflammation by sustained flow increase or decrease in rabbit carotid and femoral arteries. We observed an increase in arterial furin and MT1-MMP expression that preceded or coincided with the increase in arterial MMP2 activation during arterial remodeling. This revealed the presence of the proprotein convertase–MT1-MMP–MMP2 axis in arteries and opens a new therapeutic potential in the treatment of arterial luminal narrowing by specific proprotein convertase inhibitors.

2. Materials and methods

2.1. Animals

Fifty New Zealand White rabbits (Broekman Charles River, 3–3.5 kg) were used. The rabbits were anesthetized by intramuscular injection of methadone (0.15 ml) and vetranquil (0.15 ml) followed by intravenous injection of etomidate (1 mg/kg) and ventilation with N_2O:O_2/0.6% halothane.

To increase flow ($n = 23$), a side-to-side anastomosis was made between the artery (carotid and femoral) and vein (arteriovenous (AV) shunt). To decrease flow ($n = 24$), the artery (carotid and femoral) was partially ligated until flow was reduced to at least 60% of the initial value. Rabbits were terminated at 1, 2, 7 or 21 days after operation. At operation and termination, blood flow was measured using a transit time flow probe (Transonic System Inc.). To determine arterial inner diameter before and after the operation and at termination, an intravascular ultrasound catheter (30 MHz Du-Med, Rotterdam, The Netherlands) was placed parallel to the artery submerged in saline (i.e. extravascular ultrasound = EVUS). Before EVUS, the artery was maximally dilated by embedding for 3 min in a saline solution con-

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taining 5 mg/ml papaverin. The contra-lateral artery was used as a control. The measured arterial segments were at least 1.5 cm away from the surgical intervention area. Sham operations ($n=3$) were performed as described but without ligation or AV shunt.

The investigation conforms with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, 1985) and was approved by the ethical committee on animal experiments of the University Medical Center, Utrecht, The Netherlands.

2.2. Extraction of RNA and protein

After collection, the frozen arteries were ground with a pestle and mortar under liquid nitrogen until a fine powder was obtained. Total RNA and protein were isolated by adding 1 ml Tripure Isolation Reagent (Boehringer) to the ground artery (approximately 40 mg).

RNA and protein isolation was performed according to the manufacturer.

2.3. Zymography

Protein samples (9 μg) were separated on a sodium dodecyl sulfate–polyacrylamide gel containing 1 mg/ml gelatin (Sigma) in the 8% running gel. After running, the gel was washed 2×15 min in 2.5% Triton X-100 and incubated overnight at 37°C in Brij solution (0.05 M Tris–HCl pH 7.4, 0.01 M $CaCl_2$, 0.05% Brij 35 (Sigma)). The gel was then stained with Coomassie blue (25% methanol, 15% acetic acid, 0.1% Coomassie blue) for 1 h at room temperature (RT), followed by a destaining in 25% methanol/15% acetic acid for approximately 30 min. The different MMPs were identified by size and in comigration with its recombinant protein (rhMMP2, Accurate Chem. and Scientific Corp., NY, USA).

The amount of inactive MMP2 (72 kDa) and activated MMP2 (64 kDa) was determined using the Gel Doc 1000 system. Activated MMP2 was expressed as the relative amount of total (inactive plus active) MMP2.

2.4. cDNA synthesis and semi-quantitative PCR

Total RNA (500 ng) was converted to cDNA using the Ready to GoYou Prime system (Pharmacia) with 1 μl (200 ng) hexanucleotide according to the instructions of the manufacturer. After cDNA synthesis, the sample was diluted with DEPC-treated water to 540 μl. For PCR amplification of specific cDNAs, reactions (25 μl) contained 200 μM dNTP, 1×PCR reaction buffer (Pharmacia), 2.5 U Taq DNA polymerase (Pharmacia), 1 μM of each primer and 20 μl diluted cDNA. A typical PCR started with a 2 min incubation at 94°C, 30 s at 94°C, 30 s at 60°C or 62°C and 30 s at 72°C followed by an extension of 7 min at 72°C for 25–33 cycles. 10 μl of each reaction was electroforesed through 8% polyacrylamide gels. After running, the gel was stained with EtBr and the amount of EtBr staining of the PCR product was determined with the Gel Doc 1000 system. Control experiments were performed to determine the range of PCR cycles over which amplification efficiency remained constant and to demonstrate that the amount of PCR product was directly proportional to the amount of input cDNA. PCR amplification on total RNA which had not been reverse-transcribed showed that genomic DNA was not present (data not shown). Data are presented as relative changes in the abundance of mRNA corrected for the amount of β-actin mRNA, as an internal standard, present in the samples. The identity of the cDNA amplified was confirmed by subcloning the amplified cDNAs into PGEM-T Easy (Promega) and then sequencing the inserts (Amersham, Sequenase 2.0).

The following oligonucleotides were used as primers.

Rabbit β-actin: 5′ primer 5′-GGCATGGCTTTATTCGTGTT-3′; 3′ primer 5′-CACCTTCACCGTTCCAGTTT-3′; rabbit furin: 5′ primer 5′-CCATCCAGGCTGGTTTTGTA-3′; 3′ primer 5′-GTCCATTAAATAGAACCAACAATGC-3′; rabbit MT1-MMP: 5′ primer 5′-GTTGAATTTCCAGTATTTGTTCCC-3′; 3′ primer 5′-ACATCAAAGTGTGGGAAGGC-3′.

2.5. Immunohistochemistry

Frozen arterial segments embedded in Tissuetec (Akora) were cut into 5 μm sections and fixed for 10 min in acetone containing 0.03% H_2O_2 to block endogenous peroxidase. Sections were then incubated with 10 μg/ml mouse anti-MT1-MMP monoclonal antibody (mAb) (Campro), 10 μg/ml mouse IgG1/k or RAM11 (Dako) overnight at 4°C in phosphate-buffered saline (PBS)/bovine serum albumin (BSA) 0.1%. After overnight incubation the sections were rinsed in PBS (three times for 5 min) and incubated with 1 μg/ml horse anti-mouse biotin Ab (Dakopatts) in PBS/BSA 1% containing 1% normal rabbit serum (1 h, RT). Next, the sections were rinsed in PBS (three times for 5 min) and incubated with streptavidin peroxidase (Dako) in PBS/BSA 1% containing 1% normal rabbit serum (1 h, RT) and sequentially treated with a sodium acetate buffer containing 0.4 mg/ml 3-amino 9-ethylcarbazole substrate for 15 min.

2.6. Statistical analysis

Statistical analysis of the results was performed by the Wilcoxon matched pairs signed rank test. Data are presented as mean ± S.E.M. Differences were considered as statistically significant for P-values less then 0.05.

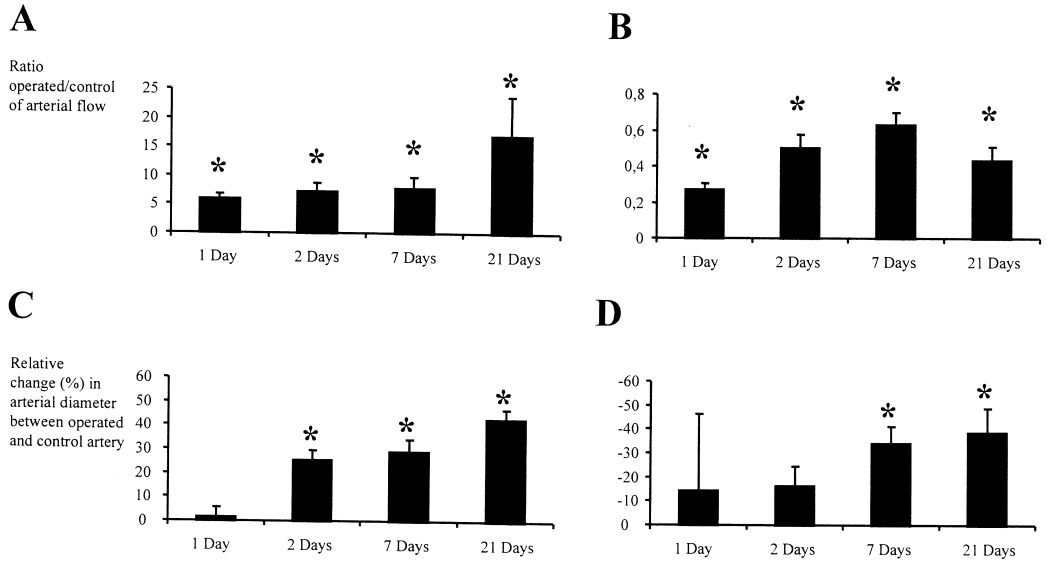

Fig. 1. Blood flow and arterial remodeling after AV shunting or partial ligation of the carotid and femoral rabbit artery 1, 2, 7 and 21 days after operation. A: Increase in blood flow after AV shunting compared to contra-lateral control artery. B: Decrease in blood flow after partial ligation compared to contra-lateral artery. C: Remodeling of the artery after flow increase. D: Remodeling of the artery after flow decrease in relative arterial diameter changes compared to the contra-lateral control artery. $n=10$–18 per time point, $^*P<0.05$ compared with control values (ratio = 1).

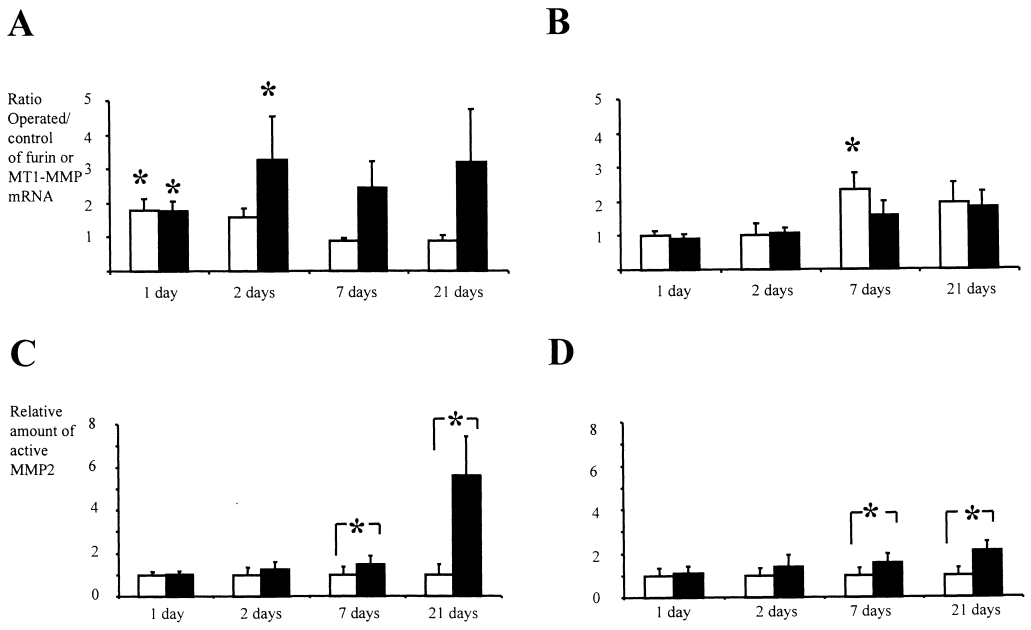

Fig. 2. Furin and MT1-MMP mRNA levels and MMP2 activation, 1, 2, 7 and 21 days after sustained change in blood flow. A: Ratio of furin (white bars) and MT1-MMP (black bars) mRNA levels between operated and contra-lateral control arteries during flow increase and (B) during flow decrease. (C) Relative amount of activated MMP2 of total MMP2 in flow-increased (black bars) and contra-lateral control arteries (white bars) and (D) in flow-decreased (black bars) and contra-lateral arteries (white bars). $n = 10$–18 per time point, $^*P < 0.05$.

3. Results

3.1. Arterial flow and remodeling after AV shunting and partial ligation

The increase in flow by AV shunting and the decrease in flow by partial ligation are depicted in Fig. 1A,B. After creating the AV shunt, flow increased progressively to 15-fold compared to the contra-lateral artery. Partial ligation resulted in a 2–3-fold decrease in both artery types. Since flow changes were similar in both arteries, the data were pooled. At all time points, the change in flow of both arteries was significant.

Changes in arterial diameter after flow increase and flow decrease are shown in Fig. 1C,D. Arterial diameter changes were similar in the femoral and carotid arteries. One day after flow increase, arterial size did not change. At 2 days after flow increase, arterial diameter increased compared to the contra-lateral artery ($25 \pm 4\%$). Arterial diameter increased further up to $42 \pm 4\%$ (Fig. 1C) at day 21. At 1 and 2 days after flow decrease, the arterial diameter did not change significantly. A significant decrease in arterial diameter was found after 7 days ($-34 \pm 7\%$) and increased slightly at 21 days ($-39 \pm 10\%$) compared to the contra-lateral artery (Fig. 1D).

3.2. Furin, MT1-MMP expression and MMP2 activation

To explore if arterial remodeling was associated with furin and MT1-MMP expression, we used semi-quantitative PCR to measure furin and MT1-MMP mRNA levels at the different time points after flow increase or decrease (Fig. 2A,B). Gelatin zymography was used to determine MMP2 activation (Fig. 2C,D). In each gelatin zymography gel, MMP2 was identified in size and in co-migration with its recombinant protein.

After sustained flow increase, furin mRNA ratios (increased flow/control) were significantly higher at 1 day (1.8), declined at day 2 (1.6), and returned to control values at days 7 and 21. MT1-MMP mRNA ratios (increased flow/control) were significantly increased at day 1 and day 2 (1.8 and 3.3 respectively) and remained high at days 7 and 21 (2.4 and 3.2 respectively). Between the flow-increased and contra-lateral control artery, the relative amount of activated MMP2 showed a significant difference at day 7 (1.5) and day 21 (5.6).

After sustained flow decrease, furin mRNA ratios (decreased flow/control) did not alter at days 1 and 2 and were only higher (2.4) after 7 days sustained decrease in blood flow. At day 21, furin mRNA ratios seemed to decline again (1.9). MT1-MMP mRNA ratios stayed also at baseline at 1 and 2 days and tended to increase at day 7 (1.6) and day 21 (1.8). Comparison of the relative amount of activated MMP2 between the flow-decreased and control artery showed a significant difference at day 7 (1.4) and day 21 (2.1). No differences were found in furin, MT1-MMP levels and MMP2 activation in the sham-operated arteries.

3.3. Localization of MT1-MMP in the arterial wall after flow change

To identify the arterial layers in which the proprotein–MT1-MMP axis is present, we stained sections of a femoral artery after flow increase with an Ab directed against MT1-MMP (Fig. 3). A strong staining was observed in the medial and adventitial layer of the artery (Fig. 3A) and no staining at all with an isotypic control Ab (Fig. 3B).

No macrophages were detected with the acid phosphatase method [18] as well as with the RAM11 Ab (results not shown).

4. Discussion

Proprotein activation by furin is important in several fundamental biological processes like cellular signaling, embryogenesis and extracellular matrix composition [19]. The finding

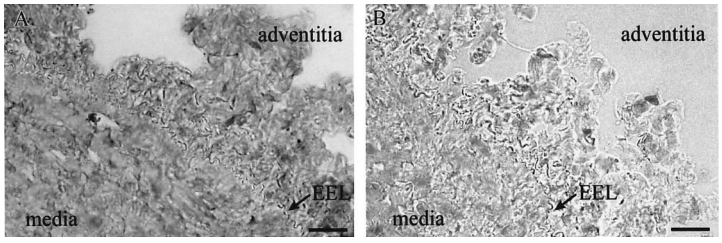

Fig. 3. A: Immunohistochemistry of rabbit femoral artery, 7 days after sustained increase in blood flow using mAb against MT1-MMP or (B) an isotypic control Ab. EEL is the external elastic lamina between the arterial medial and adventitial layer. Bar is 50 μm.

that proprotein convertase-activated MT1-MMP is the in vivo activator of MMP2 in mice [11] showed that the amount of activated MMP2 can be used to monitor MT1-MMP activation and identifies the proprotein convertases as an important target for local intervention in tissue remodeling. This idea is strengthened by the observation that MMP inhibition blocks constrictive arterial remodeling after balloon dilation [8,9]. A recent in vitro study demonstrates the existence of a proprotein convertase–MT1-MMP–MMP2 axis that can regulate extracellular matrix remodeling. Moreover, it identifies the proprotein convertases furin and PC6 as the major processing enzymes for MT1-MMP although a minor role for PC7 and proprotein convertase independent MT1-MMP activation cannot be excluded [15]. However, the role of this proprotein–MMP axis in remodeling of adult arteries, an important determinant of arterial lumen loss during atherosclerosis [4,5] and after balloon angioplasty [1–3], has not yet been described.

In this study, arterial remodeling was induced in rabbits via sustained increase or decrease in blood flow. The sustained flow increase (5–15-fold increase) resulted in arterial enlargement after 2 days sustained flow increase. Flow decrease resulted in arterial shrinkage after 7 days sustained flow decrease. Similarly, expression of furin and MT1-MMP mRNA was induced before and at the onset of arterial enlargement. At the onset of arterial shrinkage (day 7) also furin mRNA was increased. During arterial enlargement, MMP2 activation was almost doubled at day 7 and increased 6-fold at day 21, while during arterial shrinkage MMP2 activation was doubled at days 7 and 21.

The increase in furin and MT1-MMP mRNA levels also revealed that the furin–MT1-MMP axis was upregulated preceding and during arterial enlargement and shrinkage. Strong correlations are found between MT1-MMP mRNA levels and MMP2 activation [20] on one hand and furin mRNA and transforming growth factor (TGF) β1 activation on the other hand [21]. This implies that furin and MT1-MMP activity are transcriptionally regulated and that furin mRNA levels as well as MT1-MMP mRNA levels are good indicators for furin and MT1-MMP activity. Similar to the MMP2 activation and arterial diameter, the increase in furin and MT1-MMP mRNA is earlier and higher after flow increase (10–15-fold increase in blood flow) than after flow decrease (2–3-fold decrease in blood flow). This suggests that regulation of MMP2 activation in arterial remodeling depends on the degree of shear stress change although a role for the direction of arterial remodeling cannot be excluded. Shear stress dependent regulation might involve TGFβ1 which is regulated by fluid shear stress [22] and stimulates furin mRNA expression [21].

After flow increase and decrease, arterial remodeling occurs without inflammation and neointima formation and makes this an ideal model to study arterial remodeling only. MT1-MMP staining suggests that both media (smooth muscle cells) and adventitia (fibroblasts) are involved in the process of MMP2 activation. These cells are also the major collagen producing cells of the arterial wall [23] and can therefore degrade and synthesize collagen to reshape the collagen arterial skeleton.

In conclusion, these data show that the furin–MT1-MMP–MMP2 axis is upregulated before and during both modes of arterial remodeling and open the possibility to use local delivery of a synthetic furin inhibitor [24] or a protein-based proprotein convertase (furin and PC6) blocker [25] to intervene in pathological arterial remodeling.

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