Collagen turnover in arterial disease

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Contents

1 General introduction

ATHEROSCLEROSIS

Cardiovascular disease is the main cause of death and rapidly becoming the number one killer in Western societies[1]. Every year more than 19 million people worldwide experience a sudden cardiac event and atherosclerosis accounts for the majority of these events. Increased atherosclerotic plaque formation can lead, together with constrictive arterial remodeling, to lumen reduction and finally to lumen obstruction. In the initial progression of atherosclerosis, compensatory outward remodeling tends to preserve lumen area in coronary arteries [2] (Figure 1). However, due to atherosclerotic plaque rupture, abrupt occlusion of a coronary artery can also occur which is the common cause of sudden death, accounting for 70% of fatal acute myocardial infarctions and/or sudden coronary death[3-5]. Percutaneous transluminal angioplasty (PTA) or balloon angioplasty, atherectomy, stenting, and vein grafting are the approaches generally used to treat coronary, but also peripheral atherosclerotic disease. Their goal is to restore the blood supply.

ARTERIAL INJURY

The arterial repair after injury includes neovascularization, infiltration of inflammatory cells, local cell proliferation, differentiation, and apoptosis along with extracellular matrix (ECM) deposition[6-11]. The repair process or restructuring of the artery after balloon angioplasty comprises two major features: neointimal formation and geometrical remodeling. The accumulation of smooth muscle cells and/or advential fibroblasts and extracellular matrix between the endothelium and internal elastic lamina (subendothelium) of the artery is defined as neointimal formation. Geometrical remodeling is a structural change in total arterial circumference and comprises both outward as inward remodeling (shrinkage) of the artery. If neointimal hyperplasia occurs, constrictive remodeling (shrinkage) will accelerate lumen loss, whereas expansive remodeling (enlargement) will prevent the narrowing of the lumen (Figure 2)[12].

Nowadays, 70% of coronary arteries are stented after balloon angioplasty to prevent mechanically arterial shrinkage, however, stents do not decrease neointimal hyperplasia and in fact lead to an increase in the proliferative hyperplasia of restenosis. Using sirolimus-eluting stents, quantitative coronary angiography and intravascular ultrasound demonstrated a virtual complete inhibition of intimal hyperplasia at 6 and 12 months. However, it needs to be proven that current drug-eluting stents will produce similar results in 'real life' interventional practice (long lesions, lesions in small vessels, in vein grafts, chronic total occlusions, and bifurcated and ostial lesions).

During atherosclerotic lesion development, neointimal hyperplasia and arterial remodeling, the structure of the arterial wall changes dramatically. What these structural changes are, and how they are regulated has not been well described and a complete understanding of the underlying mechanisms has not been reached yet.

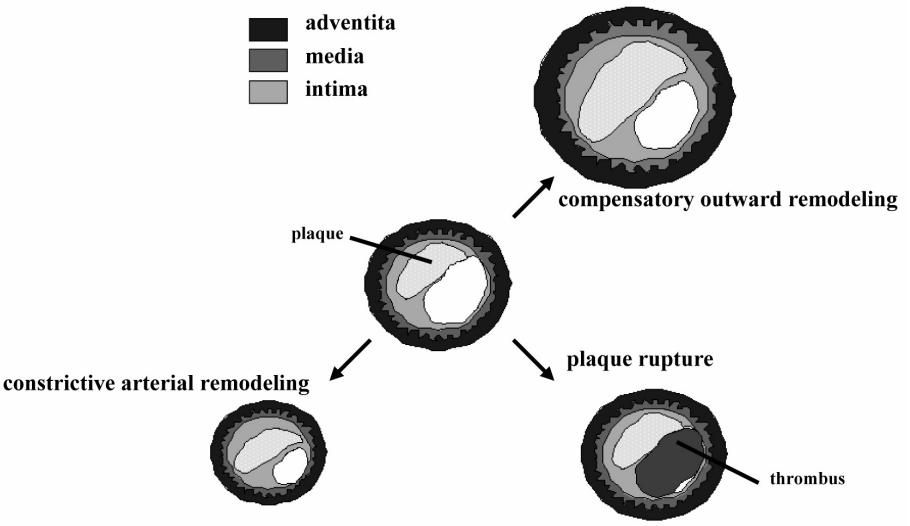

Figure 1: *Arterial plaque formation, together with constrictive arterial remodeling or plaque rupture can lead to lumen reduction or even arterial obstruction. In the initially progression of atherosclerosis, lumen area can be preserved if arteries undergo compensatory outward (expansive) remodeling.*

COLLAGEN TURNOVER

The structural shape, cellular arrangement and the tensile strength of the artery are determined by the extracellular matrix (ECM). Under normal physiological conditions, the ECM undergoes constant maintenance, with a relatively low basic turnover of its constituents. However, damage to the tissue produces a response, in order to preserve function, and increases the activity of cells in the ECM. Also in physiological vascular remodeling, a degradation and reorganization of the extracellular matrix (ECM) is needed. Even in atherosclerosis, while atheroma develops, the arterial wall undergoes major reorganizations.

In arteries, collagen is one of the major components of the extracellular matrix, which together with other proteins forms a structural protein network that determines the stiffness and size of the arterial wall. Collagen is essential to maintain the integrity of the arterial wall and for cell migration[13]. Therefore increased collagen synthesis is an important part of the repair process after injury[14-16]. The outcome, however, may be a double-edged sword: in an effort to save tissue integrity, increased deposition of collagen may impair normal functions. Also in human atherosclerotic plaques, collagen content and collagen turnover play an important role. The more fibrous lesions, containing more collagen, are thought to be more stable compared to the more prone to rupture plaques that contain less collagen deposition in their fibrous caps.

Since collagen is an essential component in structural adaptation of the arteries to physiological and pathological changes, a better understanding of collagen synthesis and degradation in cardiovascular diseases is essential if further diagnostic and therapeutic improvements are to be achieved. In this thesis, different proteins involved in the process of collagen turnover are studied in physiological and pathological models of arterial remodeling and in the human atherosclerotic plaque to improve the knowledge on collagen turnover in arterial disease and to find new possible targets to intervene in arterial remodeling.

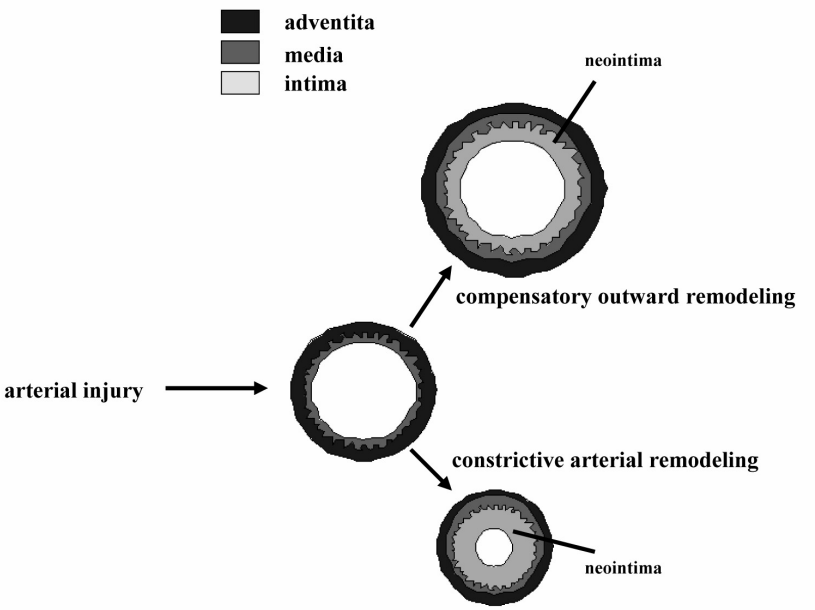

Figure 2: *A method to restore blood supply is balloon angioplasty, although long-term benefit is poor due to restenosis of the lumen. If neointimal hyperplasia occurs, enlargement of the artery (compensatory outward remodeling) will prevent lumen narrowing, whereas constrictive remodeling will accelerate lumen loss.*

COLLAGEN SYNTHESIS

Collagen production may be controlled at several levels, like transcription, mRNA stability, and the activity of enzymes involved in collagen processing. For example, Collagen type I is the product of two different genes, α1 (I) and α2 (I), which are coordinately regulated. Collagen synthesis involves a large number of co-translational and post-translational events[17]. It involves the concerted, coordinated action of a large number of essential enzymes and molecular chaperones: immunoglobulin heavy chain binding protein (BiP)[18], protein disulfide isomerase (PDI)[19], and Heat

shock protein 47 (Hsp47). Hsp47 is, in contrast to BiP and PDI, thought to be a collagen specific molecular chaperone.
Heat shock protein 47 (Hsp47) is an intracellular molecular chaperon that is essential for the maturation and secretion of newly synthesized procollagen[20-22]. Hsp47 expression always coincides with procollagen expression[23-25], whereas cells that do not produce collagen do not produce Hsp47. Collagen accumulation is suppressed in experimental glomeruloneophritis, using Hsp47 anti-sense oligonucleotides[26]. Therefore, Hsp47 is a suitable marker for collagen synthesis and a possible target to intervene in the arterial response to injury. In the endoplasmic reticulum (ER), Hsp47 binds procollagen molecules facilitating triple helical formation, however, the exact function of Hsp47 is not known. The Hsp47-procollagen complex is dissociated in the golgi-apparatus, after which Hsp47 is recycled back to the ER and procollagen is secreted out of the cell (Figure 3).
After secretion, collagen's propeptides are removed by peptidases at the cell surface. The function of the propeptides in the ECM is unknown, but they may serve as modulators of fibril growth, or constitute feedback regulation. The propeptides can be measured in almost all body fluids, including the serum[27]. Although only a small amount of procollagen is needed to maintain the collagen skeleton under steady state conditions, large amounts of procollagen are synthesized and excreted, but undergo immediate degradation[28]. After cleavage of the propeptides, tropocollagen molecules assemble spontaneously into fibrils and, due to cross linking by lysyl oxidase, a compact collagen fiber is formed. The secreted collagen molecules can be built into the collagen matrix. However, inhibition of de novo procollagen synthesis impairs cell migration, suggesting that newly synthesized procollagen is also essential for cellular migration (Figure 3)[13].

COLLAGEN DEGRADATION

The orderly degradation of interstitial extracellular matrixes and basement membranes are one of the fundamental processes involved in growth, development, morphogenesis, remodeling, and repair under both normal and pathological conditions. Matrix metalloproteinases are believed to be the main physiologically relevant mediators of matrix degradation[29]. Matrix metalloproteinases (MMPs), also called matrixins, are a family of zinc-dependent endopeptidases capable of degrading extracellular matrix components such as collagens, proteoglycans, elastin, laminin, fibronectin and other glycoproteins (Figure 3)[30]. The MMPs play important roles in embryo development[31], bone resorption[32], angiogenesis[33,34], and in diseases associated with unbalanced degradation of extracellular matrix such as arthritis[35], wound healing[36], cancer cell metastasis[37], and in atherosclerosis[38,39].
MMPs degrade the extracellular matrix during physiological and pathological remodeling, but also the biological activity of non-matrix substrates is regulated by MMP processing, such as TNF-α[40], growth factors and their receptors[41] and endothelin[42]. A clear understanding of the mechanisms governing the regulation of

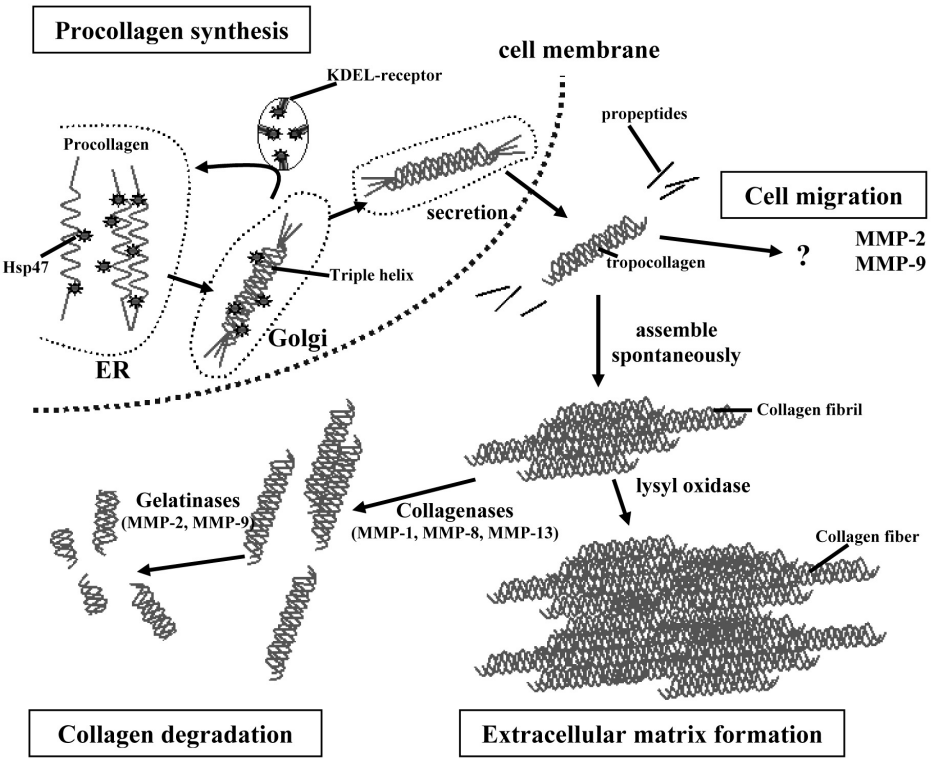

Figure 3: *In the endoplasmic reticulum (ER), procollagen synthesis is started with the formation of single procollagen molecules. Hsp47 binds these procollagen molecules and facilitates triple helical formation. After transport to the golgi-apparatus, Hsp47-procollagen complex is dissociated. Hsp47 is recycled back, via the KDEL-receptor, to the ER and procollagen is secreted out of the cell. After secretion, collagen's propeptides are removed by peptidases at the cell surface. After cleavage of the propeptides, tropocollagen molecules assemble spontaneously into fibrils and, due to cross linking by lysyl oxidase, a compact collagen fiber is formed. The secreted collagen molecules can be built into the collagen matrix, however, it is suggested that newly synthesized procollagen is also essential for cellular migration Matrix metalloproteinases (MMPs), like the collagenases and gelatinases, degrade the extracellular matrix components such as collagen. Moreover, MMPs are also involved in cellular migration.*

MMP activity during normal physiological processes should give further insights into the uncontrolled degradation occurring in degradative pathologies. Considering the potentially destructive effects of uncontrolled tissue degradation, the balance between matrix synthesis and degradation must be precisely regulated to maintain the structural integrity and function of tissues[43].

MMP activity is regulated at multiple levels: gene transcription and synthesis of inactive zymogens[44], posttranslational activation of zymogens[45], and endogenous inhibition by the tissue inhibitors of MMPs (TIMPs)[43]. MMPs are secreted in a latent,

zymogen form in which the prodomain is thought to fold over and shield the catalytic site. MMP activation can occur when the prodomain is cleaved by other proteases, such as plasmin or membrane-type MMPs (MT-MMPs)[46-48], or when the zinc-cysteine bond is interrupted[49]. Such an interruption leads to auto-activation[50].
Increased expression of several MMPs and presence of MMP activity were observed in diseased human arteries[51-53], but also in association with arterial morphological changes in experimental models of atherosclerosis and restenosis[48,54,55]. MMP expression and activation is regulated by all kind of inducers of vascular structural changes: hemodynamics[56], injury[57], inflammation[51] and oxidative stress[58].
The use of various nonselective MMP inhibitors to modify arterial restenosis after experimental balloon injury resulted in prevention of constrictive remodeling and subsequent luminal narrowing[59-61]. In addition, an initial inhibition of neointima formation has been observed. Increased cell proliferation, however, was responsible for a catch-up in neointimal area[62, 63]. The use of broad-spectrum MMP inhibitors in clinical settings revealed major side effects[64], but considering the positive outcomes on restenosis and arterial remodeling, MMP inhibitors are likely to be useful to reduce cardiovascular death. Therefore, MMP inhibitors that are more specific are needed to be able to intervene more specifically. However, due to the lack of these specific MMP inhibitors, there is little insight into the role and regulation of the individual MMPs in vascular diseases. We need to understand the process by exploring when, where, and how MMPs are synthesized and regulated.

OUTLINE OF THE THESIS

The aim of this study is to explore the mechanisms of arterial collagen turnover, which plays an important role in arterial physiological and pathological responses. Physiological arterial remodeling is studied in a rabbit model of sustained flow changes, resulting in arterial remodeling without neointimal formation. In **chapter 2**, collagen synthesis, collagen degradation and collagen fiber content are studied in this rabbit model of sustained flow changes. The matrix metalloproteinases (MMPs) are synthesized as inactive zymogens and activation is essential. The presence of the furin – mt1-mmp – mmp-2 activation cascade in the arterial wall during flow-induced outward and inward remodeling is reported in **Chapter 3**.

Pathological remodeling is studied after arterial balloon dilation, resulting in arterial shrinkage and neointimal hyperplasia. In **chapter 4**, collagen synthesis, collagen degradation and collagen fiber content is investigated in the arterial response to injury in New Zealand White rabbits. Furthermore, temporal changes are related to remodeling and a mechanism of cellular procollagen protein throughput is suggested. In **chapter 5**, furin expression is studied and the effect of its inhibition on MT1-MMP and TGF-β activation in the arterial response to injury is explored.

The expression of the extracellular matrix metalloproteinase inducer (EMMPRIN) and the association with MMP activity after arterial injury in rabbits and in human atherosclerotic lesions is described in **chapter 6**. Furthermore, the role of EMMPRIN as a potential new serum marker reflecting MMP activity in the arterial wall is studied.

In **chapter 7**, the expression of EMMPRIN and furin in human atherosclerotic plaques (Athero-express) is studied and related to plaque characteristics and MMP activity.

In **chapter 8**, the results of the studies that have been described in the preceding chapters are summarized and discussed.

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2 Increase in collagen turn-over and not in collagen fiber content is associated with flow-induced arterial remodeling

Joost P.G. Sluijter, Mirjam B. Smeets, Evelyn Velema, Gerard Pasterkamp, Dominique P.V. de Kleijn.

ABSTRACT

Background: *Degradation and synthesis of collagen are common features in arterial geometrical remodeling. Previous studies described an association between arterial remodeling and an increase in collagen fiber content after balloon injury. However, this does not exclude that the association between collagen content and remodeling depends on arterial injury since the association of collagen fiber content and arterial remodeling, without arterial injury, has not been investigated. Aim of the present study was to study the relation between flow-induced arterial geometrical remodeling, without arterial injury, and collagen synthesis and degradation, collagen fiber content and Moesin levels, which are associated with cell migration.*

Methods and results: *In 23 New Zealand White rabbits an arteriovenous shunt (AV-shunt) was created in the carotid and femoral artery to induce a structural diameter increase or a partial ligation (N=27 rabbits) to induce a diameter decrease. In both models, arterial remodeling was accompanied by increased procollagen synthesis, reflected by increased procollagen mRNA or Hsp47 protein levels. In both models, however, no changes were detected in collagen fiber content. Active MMP-2 and Moesin levels were increased after AV-shunting.*

Conclusions: *Collagen synthesis and MMP-2 activation were associated with arterial remodeling. However, a change in collagen fiber content was not observed. These results suggest that, during flow-induced geometrical arterial remodeling, increases in collagen synthesis are used for matrix collagen turnover and cell migration, and not to augment collagen fiber content.*

SUBMITTED FOR PUBLICATION

INTRODUCTION

Arterial remodeling comprises structural changes in vessel circumference varying from arterial enlargement to shrinkage. Remodeling occurs during *de novo* atherosclerosis [1;2], after balloon angioplasty [3] and also during sustained flow changes [4;5]. During arterial remodeling, degradation and resynthesis of collagen, one of the major components of the arterial wall, is important [6;7].

Inhibition of collagen degradation via matrix metalloprotease inhibitors resulted in diminished arterial remodeling after balloon angioplasty [8;9] and after sustained blood flow changes [10]. Also collagen synthesis has been associated with arterial remodeling [6;11]. The relation between collagen synthesis and collagen fiber formation is generally accepted. However, the association of collagen fiber deposition and arterial remodeling has not been investigated directly. Several studies describe an increase in collagen fiber content after arterial balloon injury [6;12-17]. In addition, two studies describe a positive correlation between collagen fiber content and arterial remodeling [13;16] while Coats et al [14] showed that collagen content is lower in restenotic vessels. This increase in collagen fiber content in the balloon injury models suggests that this is necessary for arterial remodeling. However, in these models not only remodeling but also a complete process of wound healing including neointima formation occurs, moreover, collagen content was measured after remodeling occurred. Recently, we showed in a temporal study that an increase in collagen fiber content already is evident before arterial remodeling. In addition, collagen fiber content did not change during active arterial remodeling, in contrast to collagen synthesis and degradation [11]. This suggests that collagen synthesis and degradation is needed for fine tuning or reshaping of the collagen fibers in the arterial wall [17;18] and for cell migration, essential in the process of arterial restructuring. This in contrast to the increased collagen fiber content that might reflect the arterial response to injury and is therefore associated with arterial remodeling. For this, we hypothesized that when only remodeling occurs, collagen synthesis and degradation will increase while collagen fiber content is unaltered.

In this study, we induced arterial remodeling without neointima formation and inflammation by sustained flow increases or decreases in rabbit carotid and femoral arteries. During arterial remodeling, we observed an increase in procollagen mRNA and the collagen chaperon Heat Shock Protein 47 (Hsp47) protein and Matrix Metalloprotease 2 (MMP-2) activation without subsequent changes in collagen fiber content. These results demonstrate that collagen synthesis, potentially used for matrix collagen turnover and cell migration and not collagen fiber deposition, is associated with structural arterial remodeling.

MATERIALS AND METHODS

Animals

Fifty New Zealand White rabbits (Broekman Charles River, 3-3.5 kg) were studied. 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 N2O:O2/0.6% Halothane.

To increase flow (N=23), a side-to-side anastomosis was made between the carotid artery and jugular vein and between the femoral artery and femoral vein (arteriovenous shunt, AV shunt). To decrease flow (N=27), the artery (carotid and femoral) was partially ligated until flow was reduced to at least 60% of the initial value. No flow changes occurred in the untreated left femoral artery, which served as control. 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) was placed parallel to the artery submerged in saline (i.e. extra-vascular ultrasound = EVUS). Before EVUS, the artery was maximally dilated by embedding for 3 min in a saline solution containing 5 mg/ml papaverin. No differences were found in arterial diameter before and just after the operations. The contra-lateral artery was used as a control. The measured arterial segments were proximally located at least 1.5 cm from the surgical intervention area.

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.

RNA and protein extraction

Arterial segments were harvested at least 1.5 cm proximal from the surgical intervention area, which are flow-loaded and without surgical trauma. All arteries were immediately frozen at -80ºC for RNA and protein isolation. Small parts were used for immunohistochemistry analysis and were fixed for 2h in 4% paraformaldehyde, and via 15% sucrose (O/N) embedded in Tissue Tec (Sakura).

All frozen arterial segments were crushed in liquid nitrogen. Total RNA and protein were isolated using 1ml Tripure™ Isolation Reagent (Boehringer Mannheim) according to the manufacturers' protocol.

Semi-quantitative RT-PCR

Rabbit collagen I (Forward: 5'-tgccatcaaagtcttctgc-3'; Reverse: 5'- catactcgaactggaatccatc-3') and ribosomal 18S (Forward:5'-tcaacacgggaaacctcac-3'; Reverse:5'-acaaatcgctccagcaac-3') primers were designed using the Prime program at CAOS/CAMM (Nijmegen).

First-strand cDNA was made from 200 ng total RNA by using Ready-To-Go You-Prime First-Strand Beads (Amersham Farmacia Biotech) according the manufacturers protocol. The PCR products (see below) were identified by sequencing using the

T7 sequenase version 2.0 DNA sequencing kit (Amersham Pharmacia Biotech).
All cDNA were amplified double in the I-cycler iQ™ Real Time PCR (Biorad). Each reaction tube (16.2 µl) contained 14 µl diluted cDNA, 200 µM dNTP, 1x reaction buffer (BRL) containing 1:80,000 Cybergreen (Biorad), 2,5 U Taq DNA polymerase (BRL) and 1 µM of each primer. The PCR started with 2 min at 94ºC followed by 40 cycles of: 30 sec at 94ºC, 30 sec at 56ºC (collagen I) or at 50ºC (18S) and 1 min at 72ºC; and ended with 7 min at 72ºC. Quantities were determined by comparison with known quantities of cloned collagen I and 18S PCR products representing the target mRNAs.

Western blotting and zymography
Isolated protein concentrations were determined using the Biorad DC Protein Assay (Microplate Assay Protocol). Eight µg total protein was separated on a 10% SDS-PAGE gel. The gel was transferred onto a Nitrocellulose C membrane (Amersham) and blocked using Phosphate Buffered Saline (PBS)-0.1%Tween-5% Protifar (Nutricia, Netherlands). The membrane was incubated with a mAb for Hsp47 (1:1000, Colligin; SPA-470, StressGen Biotechnologies Corp.) a mAb for Moesin (1:200, NeoMarkers) and a goat-α-mouse-HRP (1:1000, DAKO) or a pAb for pro-collagen I (1:1000, Southern Biotechnology Associates), rabbit-α-goat-biotin (1:1000, DAKO), and a streptavidin-peroxidase (1:1000) in PBS-0.1% Tween-5% Protifar. Finally the blot was exposed to a chemiluminescence substrate (NEN™ Life Science Products) and detection and analysis occurred using the ChemiXRS system (Biorad). Isotype control incubations did not reveal any signal.
Zymography was performed as described before [5]. In short, protein samples (10 µg) were separated on a 10% SDS–PAGE gel containing 1 mg/ml gelatin (Sigma). After incubation overnight at 37°C in Brij solution (0.05 M Tris–HCl pH 7.4, 0.01 M CaCl2, 0.05% Brij 35 (Sigma)), the gel was stained (25% methanol, 15% acetic acid, 0.1% Coomassie blue) and pro- and active MMP-2 bands (72kD and 64kD, respectively) were analysed using the Gel Doc 1000 system (Biorad). The different MMPs were identified by its size and in co-migration with its recombinant protein. Activated MMP-2 was expressed as the relative amount of total (active plus inactive) MMP-2.

Immunohistochemistry
5 µm slides of arterial segments were pre-incubated with 10% normal goat serum (NGoS) in PBS, followed by an incubation with the mAb against Hsp47, a mAb α-rabbit-vimentin (Sigma, Clone V9) or the mAb mouse-α-rabbit macrophage (DAKO, Ram 11) O/N in 0.1% phosphate buffered saline-1% albumin (PBSA) at 4ºC. Subsequently slides were incubated with a goat-α-mouse-HRP in 1% PBSA-1%NGoS and substrate was added (fast blue BB for the Hsp47 and AEC in dimethyl-formamide for the vimentin) until staining appeared. Slides were counter stained with haematoxin and embedded in glycin/glycerol.

Picrosirius red staining

Quantification of collagen content was performed according Perree et al [19]. In short, collagen content was measured in the three different arterial layers using picrosirius red staining and digital image microscopy with circularly polarized light. Color images of the sections and the background were recorded using two filters to avoid saturation problems: a 6% density filter for measurements on the adventitial layer and a 25% density filter for measurements on media and intima. After background subtraction, the section image was converted into a greyvalue image and regions of interest (ROI) were drawn to select the three different arterial layers. The total amount of greyvalues in each layer was measured for collagen content.

Statistical analysis

Statistical analysis of the data was performed using a Wilcoxon matched pairs signed rank sum test for the Hsp47 protein and arterial diameter or a Kruskal Wallis test followed by a ManWhitney test. Data were compared to the left femoral artery (control) and presented as mean ± the standard error of the mean. Differences with P values of < 0.05 were considered statistical significant.

RESULTS

Arterial remodeling after flow increase and decrease

Both increased and decreased blood flow in the right carotid artery was accompanied by increased blood flow in the left carotid artery. Without having operated on the left carotid artery, blood flow was increased to compensate for the diminished blood flow to the brain on the right side [20]. Since flow changes were similar in both arteries, the data were pooled. Flow increase by AV shunting and flow decrease after partial ligation is depicted in Figure 1A and B, respectively. After AV shunt, flow increased progressively to 15-fold compared to the control artery. Partial ligation resulted in a 2-3 fold decrease in both artery types. At all time points, the change in flow was significant.

For quantification of arterial remodeling, changes in arterial diameter were measured after flow increase and flow decrease and are shown in Figure 1C and D. As arterial diameter changes were similar in the femoral and carotid arteries, these arteries were pooled. One day after flow increase, arterial size did not change. At two days after flow increase, arterial diameter increased compared to the contra-lateral artery (25%±4%). Arterial diameter increased up to 42%±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%±7%) and increased slightly at 21 days (-39%±10%) compared to the contra-lateral artery (Fig. 1D).

Creating the AV-shunt increased shear stress from 22 to 93 dynes/cm2. Shear stress is reduced after 21 days but still increased (30 dynes/cm^2). After a partial ligation, shear stress decreased from 20 to 8 dynes/cm^2, but was normalized after 21 days (19 dynes/cm^2) (data not shown).

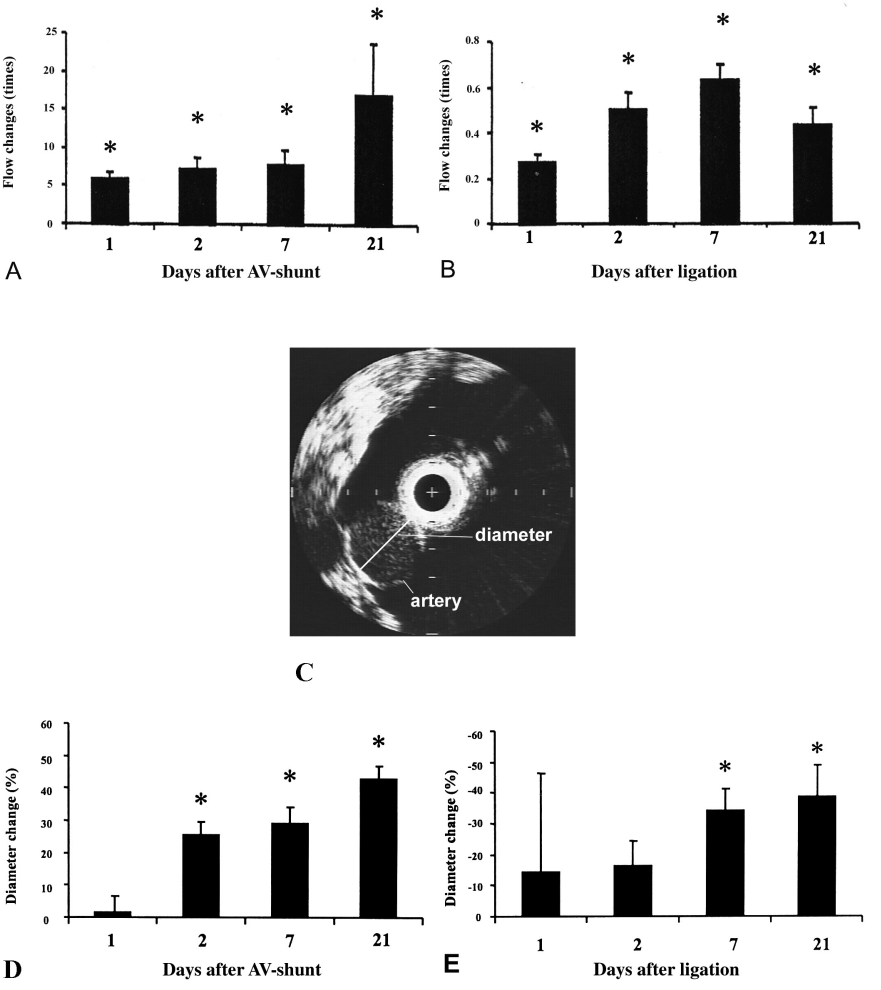

Figure 1: *Flow and diameter changes 1, 2, 7 and 21 days after arterio-venous side-to-side anastomosis (AV-shunt) or partial ligation. A: Relative increase in blood flow after AV-shunting. B: Relative decrease in blood flow after partial ligation. C: example of an ultrasound catheter image. D: Relative diameter changes (%) after AV-shunt. E: Relative Diameter changes (%) after partial ligation. [5] (Femoral and carotid arteries at each survival time are pooled. N=8-18 per time point, * = p<0.05).*

Collagen synthesis and degradation during arterial remodeling

Collagen I mRNA levels were not changed the first day after flow increase. After 2 days, collagen I mRNA was increased and remained high at day 7 and 21 (p=0.023, p=0.010, and p=0.015, respectively) (Fig. 2A). The increase in collagen I mRNA levels was not accompanied by significant increased procollagen I protein levels (Fig. 2C). However, Hsp47 protein levels were increased at 2, 7, and 21 days (p=0.012,

p=0.030, and p=0.012, respectively) (Fig.2E). Next to an increase in collagen synthesis, there was also an increase in MMP-2 activation with increased levels of active MMP-2 at 7 and 21 days (Fig.2G).
During flow decrease, collagen mRNA increase started later and was significant at day 7 (p=0.006). At 21 days, the procollagen mRNA levels were back to baseline levels (Fig.2B). Procollagen I protein levels were not increased at all time points (Fig. 2D). Hsp47 protein levels were increased at day 2 and 7 (p=0.004, p=0.006, respectively) and returned also to baseline levels at day 21 (Fig.2F). Active MMP-2 levels were increased at day 7 and 21 (Fig. 2H).
Immunohistochemistry of arteries that underwent changes in blood flow showed that Hsp47 protein was primarily located in cells of the adventitial layer (blue/black) (Fig. 3A). Double staining with α-Hsp47 (blue/black) and α –vimentin (red/brown) identified these cells as fibroblasts (Fig. 3B). No neointima and macrophages were found in the flow changed arteries, confirming that this model was not influenced by either intima formation or inflammation (data not shown).

Collagen fiber content after arterial remodeling
Using picrosirius red staining, we quantified the collagen content of the arteries after 21 days of sustained flow increase or decrease (Fig. 4). After flow increase, there were no differences between the operated and control arteries in the adventitial (black) and medial layers (grey) in collagen content (Fig.4A) and collagen density (data not shown). Also 21 days after flow decrease, there were no differences in both the adventitial (black) and medial (grey) layers between operated and control arteries (Fig. 4B).

Moesin expression during arterial remodeling
Blindt et al. described that cells transfected with Moesin revealed an increased migrative and invasive potential. We used Moesin levels as an indicator for cell migration, since increased Moesin levels are associated with an increased potential of SMCs to migrate [11; 21]. We found increased levels of Moesin 2 and 7 days after flow increase (p=0.014 and p=0.029, respectively) (Fig.4C). After flow decrease there was a tendency to increased Moesin levels at 7 days, but this was not significant (Fig.4D).

DISCUSSION

The mechanisms underlying the processes of arterial remodeling and intimal formation are not well understood. Collagen degradation and resynthesis are important for restructuring of the artery, including the arterial collagen fibers, and for cell migration. However, the association of collagen fiber formation and arterial remodeling is unclear. Cheema et al. [16] and Lafont et al. [13] suggested a role of collagen accumulation in all arterial layers in late inward remodeling. However, Coats [14] reported that increased collagen content was associated with outward remodeling and thus

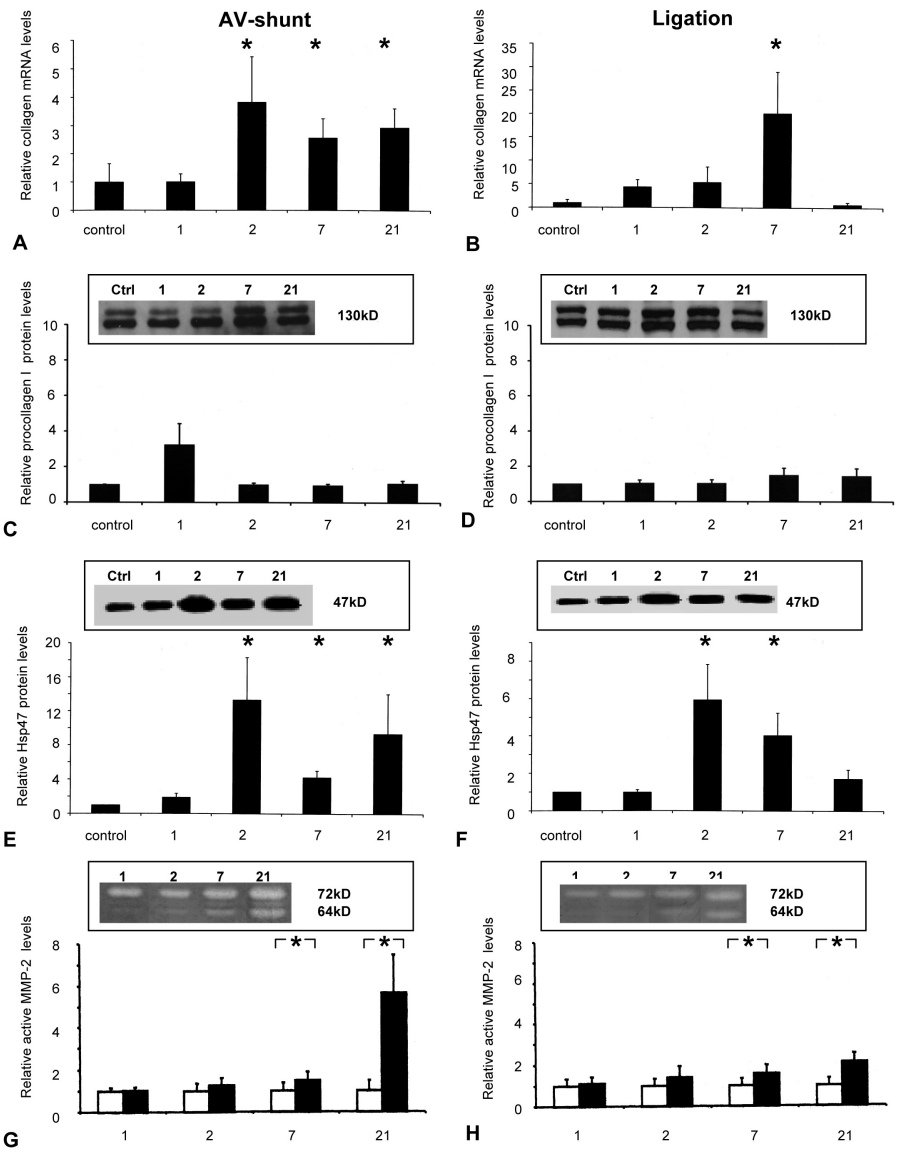

Figure 2: *Relative expression patterns 1, 2, 7, and 21 days after AV-shunt or partial ligation. Relative procollagen mRNA levels (A + B), relative Hsp47 protein levels (C + D) (Data are presented as operated arteries compared to control femoral arteries ± sem), and relative active MMP-2 (E + F) levels after AV-shunt or ligation, respectively. White bars represent relative amount of active MMP-2 of total MMP-2 in control arteries and black bars in operated arteries. (Representative Western blots and zymograms are plotted)(N=8-18 per time point, * = p<0.05)*

prevention of restenosis. In these studies, balloon injury was performed resulting in remodeling and neointima formation.
In the present study, arterial remodeling was induced in rabbits via sustained increase or decrease in flow, resulting in a structural diameter increase or decrease, respectively, without an inflammatory response or neointimal formation. After creating an AV-shunt, we observed a dramatic structural increase in arterial diameter, which was quicker as previous reported [22, 23], and probably caused by a larger increase in blood flow rates.
After flow increase, procollagen synthesis, represented by procollagen mRNA and Hsp47 protein expression, was increased at 2, 7, and 21 days and associated with outward remodeling of the artery. Procollagen I protein levels did not increase significant, however, we previously suggested a higher throughput of free procollagen after arterial injury [11] creating a constant level of free procollagen I protein levels. In both models of sustained flow changes, we did not observe a change in procollagen I protein levels either, suggesting that increased throughput of free procollagen also occurred after sustained flow changes. To confirm that changes in procollagen protein levels occur, we used Hsp47 protein expression, which is a specific collagen molecular chaperon, essential for correct folding and maturation of newly synthesized procollagen.

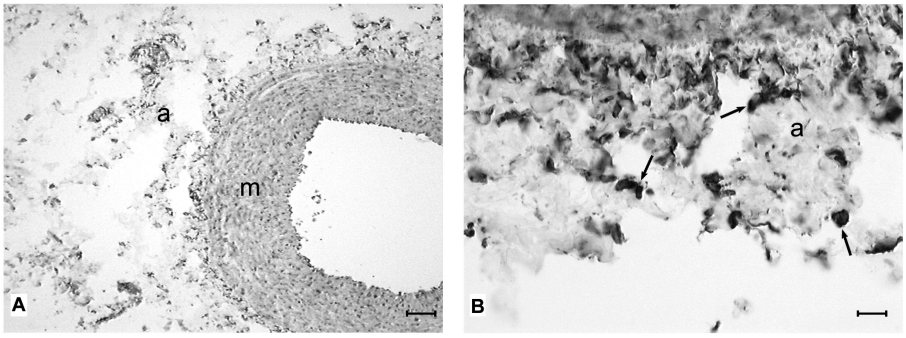

Figure 3: A: *Localization of Hsp47 in arterial cross section (blue/black), 2 days AV-shunting (bar= 500 μm).* ***B:*** *Double staining for Hsp47 and vimentin (bar= 125 μm). (a= adventitia, m= media, arrows are positive cells for Hsp47 (blue/black) and vimentin (red/brown).*

Next to the increased collagen synthesis, also the active MMP-2 levels were increased. Several papers previously described the role of MMP-2 in remodeling after flow increase. Our results confirmed data of Karwowski et al. [23], they found increased MMP-2 activity from 7 up to 21 days, however, Tronc and co-workers found early increases in MMP-2 at day 3 but these were still increased at day 15 [22]. Collagen degradation is a two-step process, the first cleavage is performed by collagenases, like MMP-1 and MMP-8, after which gelatinases, like MMP-2, degrades the remainder. Although collagen degradation is needed for arterial remodeling [8-

10], we found that increased MMP-2 levels were delayed compared to the remodeling response. In a previous study [5], we found an early increase in MT1-MMP levels (day 2) after flow increase. Moreover, Sho et al. [24] found an early increase in MMP-9 expression followed by more long term increased MMP-2 expression, indicating that not MMP-2 but other MMPs are associated with outward remodeling.
Because we used zymography, we cannot exclude that increased TIMP expression will counter balance the increased MMP activity. However, as described by Sho et al.[24] there is a disproportional increase in MMP activity compared to TIMP-2 levels after flow increase.
Also after a partial ligation of the artery, increased procollagen synthesis was accompanied by increased MMP-2 activity and associated with inward arterial remodeling.
The present study confirmed that arterial remodeling without arterial injury is associated with increased collagen synthesis and degradation, but without any increase in collagen deposition. Also other researchers have reported that geometric adaptation of the human artery to chronic increases in blood flow does not result in arterial wall hypertrophy, but rather in remodeling [25]. This is also in accordance with the data of Marijianowski et al.[26]. They showed that myocardial remodeling in humans after infarction is not associated with fibrosis of non-infarcted myocardium. However, Tronc and co-workers found increased medial cross-sectional area after flow increase [27] which is confirmed by Driss et al [28]. The latter found also increased medial collagen and elastin content, but suggested that increase in shear stress induces expansive remodeling, whereas increase in tensile stress is responsible for medial hypertrophy and fibrosis. In our model, we could not find medial hypertrophy, suggesting that only shear stress is responsible for remodeling in our model. Our data suggest that the increases found in collagen synthesis and degradation are needed for reshaping or remodeling of the artery and for cell migration, which is an important feature in arterial remodeling.
Langille et al [29] described that chronic diameter changes after flow decrease are achieved without net changes in the major constituents of the vessel media, suggesting that remodeling is accomplished through a reorganization of wall constituents. We confirmed these findings by means of collagen fiber content, and extended the data with adventitial measurements and we measured collagen synthesis and degradation that suggest an increased collagen turnover. Moreover, we showed that Moesin, a protein associated with smooth muscle cell migration, was increased during outward and inward arterial remodeling and associated with the increased procollagen expression. Blindt et al [21] described increased Moesin expression in neointimal SMCs and the increased invasive potential after Moesin transfection. Although we found a significant increase in Moesin expression after flow increase, there was just a tendency to increase after flow decrease. We interpreted the increase of Moesin expression as an increased potential of SMCs to migrate. These increases in Moesin levels were similar as in our previous observations [11] after arterial injury. After arterial injury, cell migration is a well described feature but gives only a small increase in Moesin levels.

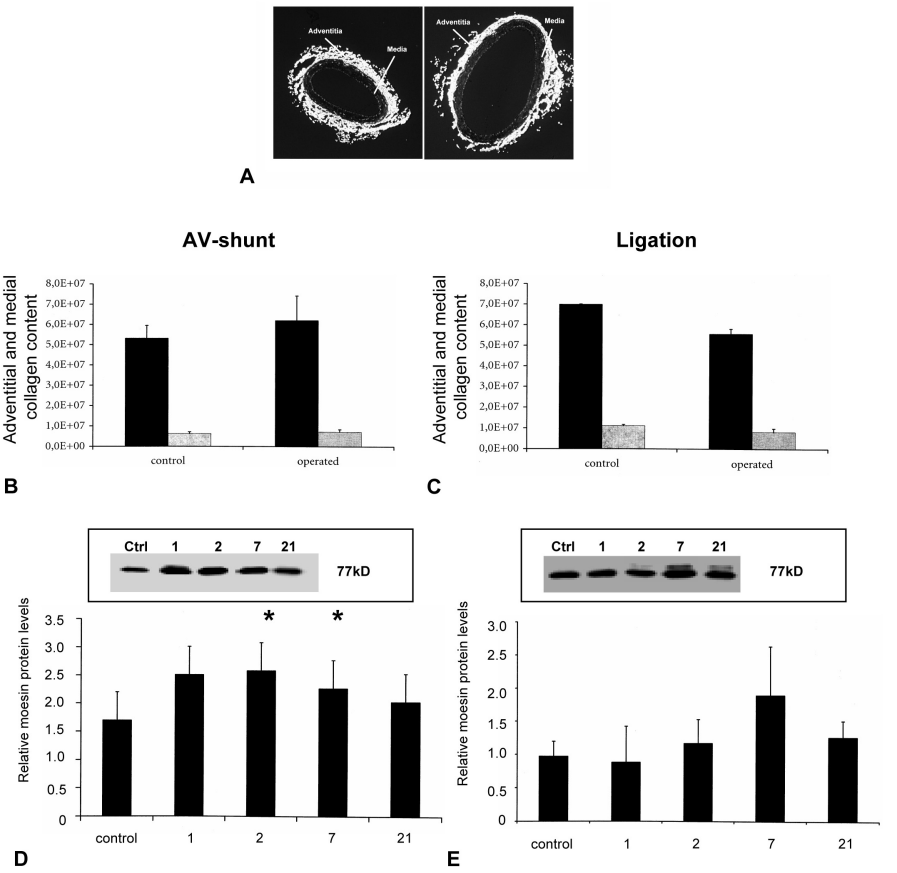

Figure 4: *Advential and medial collagen content and relative Moesin protein levels after AV-shunting or partial ligation. (A) Representative picture of picrosirius red stained sections with circularly polarized light after AV-shunt (21 days). Adventitial and medial collagen content after AV-shunting (B) and partial ligation (C). Black bars represent adventitial collagen content, gray bars represent medial collagen content. (Data are presented as mean total number of pixels per layer ± sem, N=2-5). Relative Moesin protein levels after AV-shunt (D) or partial ligation (E) (Representative Western blots are plotted)(Data are presented as operated arteries compared to control femoral arteries ± sem, N=8-18 per time point, * = p<0.05)*

We do not conclude that, in the arterial response to injury, collagen fiber turnover is not essential for arterial remodeling. Next to the papers, [13;14;16] describing an association between collagen fiber content and remodeling, Sierevogel et al. [15] found a decrease in adventitial collagen fiber content and a decrease in arterial remodeling after MMP inhibition, and Spears et al. [30] found that inhibition of the collagen fiber cross linker lysyl oxidase results in less remodeling. Although we did not measure actual collagen fiber turnover, we assume that in the process of arterial remodeling a reorganization of the collagen fibers is needed to maintain the integri-

ty of the arterial wall. However, we hypothesized that after arterial balloon injury the balance between collagen fiber synthesis and breakdown is disturbed. This result in an increase of collagen fiber deposition that might result in more inward remodeling since, among others, more cross linking can occur. Based on the data in this study, we conclude that for flow-induced remodeling this increase in absolute collagen fiber deposition is not necessary, but it remains to be investigated in the arterial response to injury.

The parameters studied in collagen turnover were increased in both models, however, the direction of remodeling was different. In both models, the artery is trying to restore shear stress levels and in both models collagen turn-over occurs. This suggests that collagen turn-over is not involved in the direction of remodeling. The direction of remodeling might be regulated by shear stress dependent factors like eNOS [31], endothelin [32] or other factors with shear stress responsive elements [33,34] but needs more investigation.

In conclusion, we report that collagen synthesis and MMP activation is associated with arterial remodeling, without an increase in collagen fiber content. This shows that an increase in arterial remodeling can occur without absolute increases in collagen fiber content and suggests that increased collagen synthesis and degradation is sufficient for arterial restructuring and cell migration in the flow-induced arterial remodeling process.

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

Dominique P.V de Kleijn, Joost P.G. Sluijter, Jenny Smit, Evelyn Velema, Wietske Richard, Arjan H. Schoneveld, Gerard Pasterkamp, Cornelius Borst.

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 MMP-2. 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.

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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 matrix metalloproteinase (MT1-MMP) is a membrane-anchored MMP and has a pivotal function in connective tissue metabolism [10,11] and activation of proMMP-2 [12].

MT1-MMP is activated after cleavage by a proprotein convertase [13,14] and activation results in a stimulation of proMMP-2 cleavage generating the activated MMP-2. In tissues from MT1-MMP null mice, activation of MMP-2 was deficient suggesting that MT1-MMP is essential for its activation in vivo [11].

In vitro studies show that the proprotein convertase - MT1-MMP - MMP-2 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 MMP-2 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 MMP-2 activation during arterial remodeling. This revealed the presence of the proprotein convertase – MT1-MMP – MMP-2 axis in arteries and opens a new therapeutic potential in the treatment of arterial luminal narrowing by specific proprotein convertase inhibitors.

MATERIALS AND METHODS

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 N2O:O2/0.6% Halothane.

To increase flow (N=23), a side-to-side anastomosis was made between the artery (carotid and femoral) and vein (arteriovenous shunt, 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) was placed parallel to the artery submerged in saline (i.e. extra-vascular ultrasound = EVUS). Before EVUS, the artery was maximally dilated by embedding for 3 min in a saline solution containing 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.

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 was isolated by adding 1 ml Tripure Isolation Reagent (Boehringer) to the ground artery (appr. 40 mg). RNA and protein isolation was performed according to manufacturer.

Zymography

Protein samples (9 µg) were separated on a SDS polyacrylamide gel containing 1 mg/ml gelatin (Sigma) in the 8% running gel. After running, the gel was washed 2 x 15 min. in 2.5% Triton X-100 and incubated O/N at 37 ^{o}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 hr. at RT, followed by a destaining in 25% methanol/15% acetic acid for appr. 30 min. The different MMPs were identified by size and in co-migration with its recombinant protein (rhMMP2, Accurate Chem. & Scientific Corp. NY).

The amount of inactive MMP2 (72 kD) and activated MMP2 (64 kD) was determined using the Gel Doc 1000 system. Activated MMP-2 was expressed as the relative amount of total (inactive plus active) MMP-2

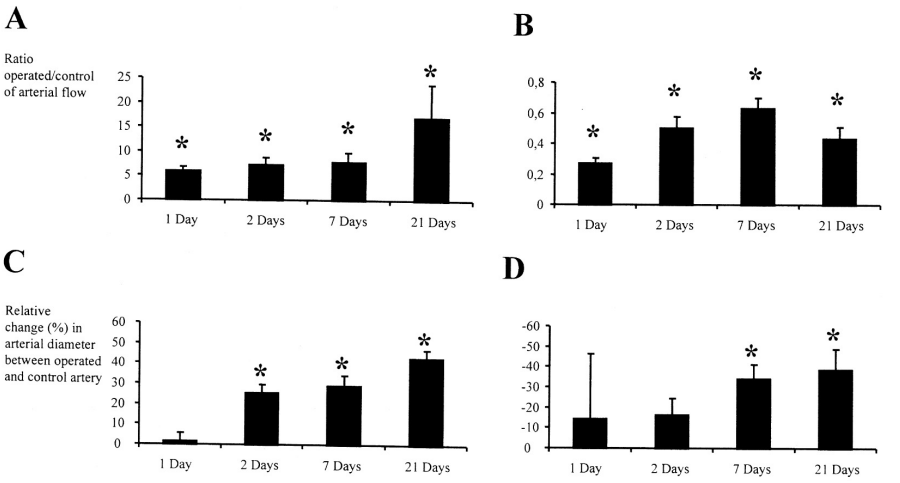

Fig. 1. *Blood flow and arterial remodeling after arteriovenous (AV) shunting or partial ligation of the carotid and femoral rabbit artery 1, 2, 7 and 21 days after operation. A: Increase in blood flow after AV shunting compared to contra-lateral control artery B: Decrease in blood flow after partial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

cDNA synthesis and semi-quantitative PCR

Total RNA (500 ng) was converted to cDNA using the Ready to GoYou Prime system (Pharmacia) with 1 ml (200 ng) hexanucleotide according to the instructions of the manufacturer. After cDNA synthesis, the sample was diluted with DEPC treated water to 540 ml. For PCR amplification of specific cDNAs, reactions (25 ml) contained 200 mM dNTP, 1 x PCR reaction buffer (Pharmacia), 2.5 U Taq DNA polymerase (Pharmacia), 1 mM of each primer and 20 ml diluted cDNA. A typical PCR started with a 2 min incubation at 94 ^{O}C, 30 sec. at 94 ^{O}C, 30 sec. at 60 ^{O}C or 62 ^{O}C and 30 sec. at 72 ^{O}C followed by an extension of 7 min. at 72 ^{O}C for 25-33 cycles. Ten ml 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 reversed 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 b-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 b-actin:
5' primer 5' GGCATGGCTTTATTCGTGTT 3'
3' primer 5' CACCTTCACCGTTCCAGTTT 3'
Rabbit Furin:
5' primer 5' CCATCCAGGCTGGTTTTGTA 3'
3' primer 5' GTCCATTAAATAGAACCAACAATGC3'
Rabbit MT1-MMP:
5' primer 5' GTTGAATTTCCAGTATTTGTTCCC 3'
3' primer 5' ACATCAAAGTGTGGGAAGGC 3'

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

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

RESULTS

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 Figure 1 A&B respectively. After creating the AV shunt, flow increased progressively to 15-fold compared to the contralateral artery. Partial ligation resulted in a two to threefold 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 Figure 1 C&D respectively. Arterial diameter changes were similar in the femoral and carotid arteries. One day after flow increase, arterial size did not change. At two days after flow increase, arterial diameter increased compared to the contra-lateral

artery (25%±4%). Arterial diameter increased further up to 42%±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%±7%) and increased slightly at 21 days (-39%±10%) compared to the contra-lateral artery (Fig. 1D).

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).

After sustained flow increase, furin mRNA ratio's (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 ratio's (increased flow/control) were significantly increased at day 1 and day 2 (1.8 & 3.3 respectively) and remained high at day 7 and 21 (2.4 & 3.2 respectively). Between the flow increased and contralateral control artery, the relative amount of activated MMP-2 showed a significant difference at day 7 (1.5) and day 21(5.6).

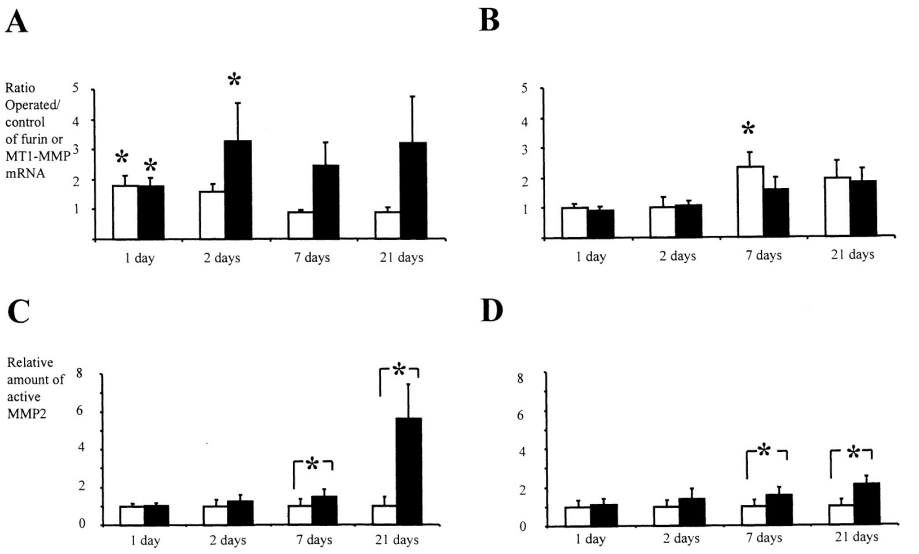

Fig. 2. *Furin and MT1-MMP mRNA levels and MMP2 activation, 1, 2, 7 and 21 days after sustained change in blood flow. A: Ratio of furin (white bars) and MT1-MMP (black bars) mRNA levels between operated and contra-lateral control arteries during flow increase and B: during flow decrease. C: Relative 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 timepoint, *=P<0.05.*

After sustained flow decrease, furin mRNA ratio's (decreased flow/control) did not alter at day 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 ratio's 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 MMP-2 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 MMP-2 activation in the sham operated arteries.

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 antibody 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 antibody (Fig. 3B).

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

DISCUSSION

Proprotein activation by furin is important in several fundamental biological processes like cellular signaling, embryogenesis and extracellular matrix composition [19]. The finding that furin activated MT1-MMP is the in vivo activator of MMP-2 in mice [11] showed that the amount of activated MMP-2 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 – MMP-2 axis that can regulate extracellular matrix remodeling [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, MMP-2 activation was almost doubled at day 7 and increased 6 fold at day 21, while during arterial shrinkage MMP-2 activation was doubled at day 7 and 21.

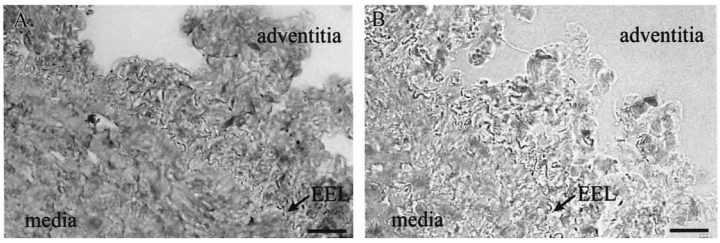

Fig. 3. *A: Immunohistochemistry of rabbit femoral artery, 7 days after sustained increase in blood flow using mAb against MT1-MMP (red) or B: an isotypic control antibody. EEL is the external elastic lamina between the arterial medial and adventitial layer. Bar is 50 μm.*

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 MMP-2 activation [20] on one hand and furin mRNA and TGFβ1 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 MMP-2 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) then after flow decrease (2-3 fold decrease in blood flow). This suggests that regulation of MMP-2 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 MMP-2 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 arterial remodeling and opens the possibility to use local delivery of a synthetic furin inhibitor [24] or a protein-based proprotein convertase blocker [25] to intervene in pathological arterial remodeling.

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4 Increased collagen turnover is only partly associated with collagen fiber deposition in the arterial response to injury

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ABSTRACT

Objective: *In the arterial response to injury collagen breakdown is studied extensively, but little is known on collagen synthesis and fiber formation. Here, we studied in vivo collagen synthesis and collagen fiber content in relation to collagen breakdown in a time course after arterial balloon injury.*

Methods and Results: *Twenty-five New Zealand White rabbits were balloon dilated in femoral and iliac arteries and terminated at 2, 7, 14 and 28 days. From day 7, both constrictive arterial remodeling and intimal hyperplasia were observed. Collagen degradation, synthesis and fiber content were studied using zymography, quantitative Polymerase Chain Reaction, western blotting and picrosirius red staining. Collagen synthesis, reflected by procollagen I and Hsp47 expression, showed an increase starting at day 2 with a maximum at day 14 and was accompanied by increased collagen breakdown as reflected by matrix metalloproteinase-1 and 2 levels. In contrast, collagen content in media and adventitia only increased between 2 and 7 days after balloon injury.*

Conclusions: *In the first week after arterial injury, increased collagen content is associated with increased collagen synthesis and degradation. However, after 1 week collagen turnover remains high in contrast to increased collagen fiber content suggesting that after 1 week, collagen turnover is used for other processes like cell migration and arterial remodeling.*

CARDIOVASCULAR RESEARCH 61 (2004) 186-195

INTRODUCTION

Arterial lumen loss after injury is determinated by neointima formation and arterial remodeling. Arterial remodeling comprises structural changes in vessel circumference varying from arterial enlargement to shrinkage and occurs during de novo atherosclerosis [1,2], after balloon angioplasty [3] and also during sustained blood flow changes.[4,5] Degradation and synthesis of collagen I, one of the major matrix proteins of the arterial wall, is an important process during arterial remodeling and neointima formation. Strauss showed an increase in collagen synthesis and matrix metalloproteinase activity shortly after a second injury. [6, 7]

Recently, inhibition of collagen degradation via matrix metalloprotease inhibitors resulted in diminished arterial remodeling after balloon angioplasty [8,9] and after sustained blood flow changes, [10] and initial reduction of neointima formation.[11] However, little is known about the regulation of collagen synthesis after arterial injury.

During collagen synthesis heat shock protein 47 (Hsp47), an intracellular molecular chaperone, binds procollagen molecules in the endoplasmic reticulum (ER) and facilitates triple helical formation.[12-14] The Hsp47-procollagen complex is dissociated in the golgi-apparatus, after which Hsp47 is recycled back to the ER and procollagen is secreted out of the cell. Hsp47 expression always coincides with procollagen expression [15-17] and is therefore a suitable marker for collagen synthesis and a possible target to intervene in the arterial response to injury.

After secretion, the N- and C-propeptides of procollagen are proteolytically cleaved after which the mature triple-helical collagen molecules assemble into multimeric fibrillar aggregates.[18] Besides fiber formation, it was found that de novo collagen synthesis is necessary to maintain cell migration [19] which is an important feature of remodeling [20] and neointima formation [11] after arterial injury.

Since little is known on *in vivo* collagen synthesis and collagen fiber formation and their association with collagen degradation after arterial injury, we used a rabbit balloon injury model to study collagen synthesis (procollagen I and Hsp47), collagen degradation (MMP-1 and 2) and collagen content over time. Furthermore, we studied Hsp47 and procollagen I levels *ex vivo* to confirm the *in vivo* results and crosslinking was performed *in vitro* to preserve the intracellular Hsp47-procollagen I protein binding. To explore the time course in which cell migration occurred, we studied moesin expression.

Here, we report that *in vivo* changes in collagen synthesis, including the increased Hsp47-procollagen I binding, and breakdown are associated only with the increase in collagen content in the first week after balloon injury. The continued increase of collagen synthesis and breakdown after one week is associated with remodeling and increased moesin expression, suggesting that collagen turnover after arterial injury is, in addition to collagen fiber deposition, also involved in other processes important in neointima formation and arterial remodeling.

MATERIALS AND METHODS

Animals

Twenty-five New Zealand White rabbits (Broekman Charles River, 3-3.5 kg) were anesthetized by methadone (0.15 ml) and vetranquil (0.15 ml) followed by etomidate (1 mg/kg) and ventilation with N_2O:O_2/0.6% halothane. Animals were housed to conform to the Guide for the care and Use of Laboratory Animals (NIH publication No.85-23, 1985) and all experiments were approved by the ethical committee on animal experiments of the University Medical Center, Utrecht.

Femoral and external iliac arteries of twenty-five rabbits were balloon dilated unilaterally with a 3.0 mm balloon 3 times for 30 seconds. We measured arterial lumen diameter at termination and post-dilation by angiography, with the use of nitroglycerin (200nM) to avoid arterial spasm, and measured the intima area by histological cross section analysis. The lumen diameter was recalculated into a lumen area. The change in internal elastic laminae (IEL) area between post-dilation and at termination is considered as remodeling of the artery. The angiographical measured lumen area post-dilation is equal to the IEL area, since no neointima is present. The IEL area at termination is calculated by summating the angiographical lumen area and the histological intima area. The relative IEL area change and intima area is calculated by dividing it with the total IEL area.

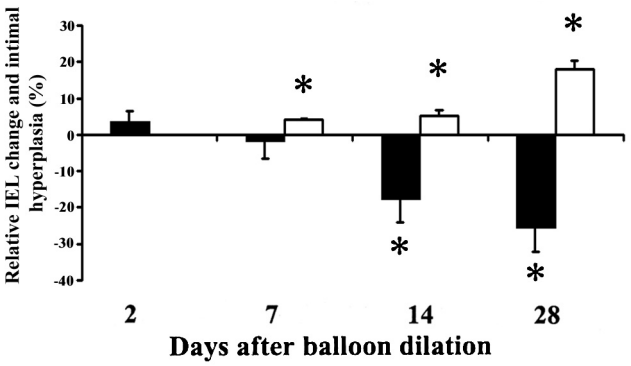

Figure 1:
*Relative internal elastic laminae (IEL) change (black) and intimal hyperplasia (white) 2, 7, 14 and 28 days after balloon dilation. IEL area change and intima are plotted as a percentage of the total IEL area (N=6-8 rabbits per time-point, *= p<0.05)*

The balloon dilated segments and the contralateral control arteries were harvested after 2, 7, 14 and 28 days (N=6-7 rabbits per timepoint). Because both femoral and iliac arteries reacted similarly, data were pooled. All arteries were harvested and immediately frozen at -80ºC for RNA and protein isolation. Small parts were used for immunohistochemistry analysis and were fixed for 2h in 4% paraformaldehyde, and via 15% sucrose (O/N) embedded in Tissue Tec (Sakura).

RNA and protein extraction

All frozen arterial segments were crushed in liquid nitrogen. Total RNA and protein were isolated using 1ml Tripure™ Isolation Reagent (Boehringer Mannheim) according to the manufacturers protocol.

Cell and tissue culture

In vitro: A cell line of vascular smooth muscle cells (v-SMC; CRL-1999, ATCC) was cultured and divided into two equal amounts of cells of which one was cross-linked as described before with succinimidylpropionate (DSP). [21] Protein was isolated after the final wash step.

Ex vivo: The distal parts of two rabbit aortas were balloon dilated as described above, the proximal parts were used as controls. Aortic rings were cultured (MEM, 10%FCS) with and without stimulation of TGF-β1 (10 ng/ml). After 7 days, rings were immediately frozen and total protein was isolated.

Quantitative RT-PCR

Rabbit Hsp47 (Forward: 5'-acctcaggcagcttgccg-3'; Reverse: 5'-aacactccaacatcaacttcc-3'), rabbit collagen type I (Forward: 5'-tgccatcaaagtcttctgc-3'; Reverse 5'-catactcgaactggaatccatc-3') and ribosomal 18S (Forward:5'-tcaacacgggaaacctcac-3'; Reverse:5'-acaaatcgctccagcaac-3') primers were designed using the Prime program at CMBI (Nijmegen).

First-strand cDNAs were produced using 200ng RNA and Ready-To-Go You-Prime

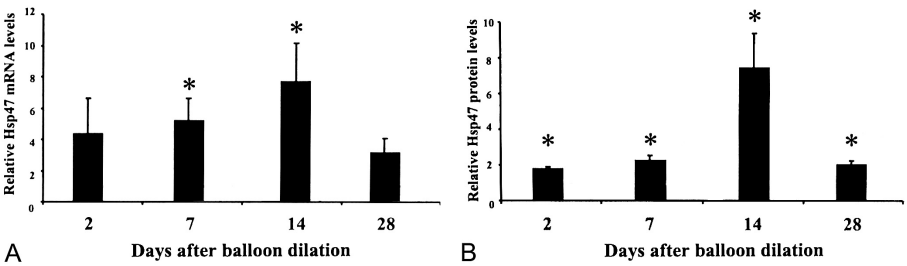

Figure 2: *Hsp47 expression patterns 2, 7, 14 and 28 days after balloon dilation. Relative Hsp47 mRNA (A) and Hsp47 protein (B) expression of balloon dilated arteries compared to contralateral control arteries.. (Data are presented as the ratio operated / control mean ± sem; femoral and iliac arteries of each survival time are pooled. N=6-8 rabbits per time point, * = P<0.05).*

First-Strand Beads (Amersham Pharmacia Biotech). cDNA was amplified in the I-cycler iQ™ Real Time PCR (Biorad) in duplicate. Each reaction contained 14 µl cDNA, 200 µM dNTP, 1x reaction buffer (BRL) containing 1:80,000 Cybergreen (Biorad), 2.5 U Taq DNA polymerase (BRL) and 1 µM of each primer. The PCR reactions started with 2 min at 94ºC followed by 40 cycles of: 30 sec at 94ºC, 30 sec at 62ºC (Hsp47), 56ºC (collagen I) or at 50ºC (18S) and 1 min at 72ºC. Quantities were determined by comparison with known quantities of cloned Hsp47, collagen I and 18S PCR products. 18S was used as internal standard. Specificity of amplification for the detection with Cybergreen is visually checked on PAGE gels. Data are presented as the ratio between the dilated and control arteries.

Western blotting and Zymography

For Western blotting, 8 μg total protein was separated on a 10% SDS-PAGE gel, transferred onto a Nitrocellulose C membrane (Amersham) and blocked using Phosphate Buffered Saline (PBS)-0.1%Tween-5% Protifar (Nutricia, Netherlands). The membrane was incubated with a mAb for Hsp47 (1:1000) (StressGen Biotechnologies Corp.), a mAb for moesin (1:200) (NeoMarkers) and a goat-α-mouse-HRP (1:1000, DAKO) or a pAb goat-α-human collagen I (1:1000)(Southern Biotechnology Associates Inc), rabbit-α-goat-biotin (1:1000, DAKO) and a streptavidin- peroxidase (1:1000) in PBS-0.1%Tween-5% Protifar. The polyclonal antibody used for collagen I showed two bands representing the α(I)-chains (1 and 2) and at the bottom the α(I)-chains without the pro-peptides, representing the free procollagen molecules.
Chemiluminescence substrate (NEN™ Life Science Products) and the Kodak X-Omat exposure™ Blue XB-1 films were used for detection; the bands were analyzed using the Gel Doc 1000 system (Biorad). Isotype control incubations did not reveal any signal.
To treat rabbit protein samples with collagenase A (Roche), 15 μg of total protein was incubated with 2 mM PMSF, 1 mM $CaCl_2$, 50 mM M Tris-HCL (pH7.5) and 0.05 U collagenase A at 37 °C for 30 min. Subsequently, Western blotting was performed as described above.
Zymography was performed as described before.[5] In short, protein samples (10 μg) were separated on a 10% SDS–PAGE gel containing 1 mg/ml gelatin (Sigma) or 2 mg/ml casein (sodium salt, Sigma) in the running gel. After incubation overnight at 37°C in Brij solution (0.05 M Tris–HCl pH 7.4, 0.01 M CaCl2, 0.05% Brij 35 (Sigma)), the gel was stained (25% methanol, 15% acetic acid, 0.1% Coomassie blue) and MMP-2 (gelatin) and MMP-1 (casein) bands were analysed using the Gel Doc 1000 system (Biorad).

Immunohistochemistry

5 μm sections of arterial segments were pre-incubated with 10% normal goat serum (NGoS) in PBS, followed by an incubation with the mAb against Hsp47, a mAb α-rabbit-vimentin (Sigma, Clone V9) or the mAb mouse-α-rabbit macrophage (DAKO, Ram 11) O/N in phosphate buffered saline-1% albumin (PBSA) at 4ºC. Subsequently, sections were incubated with a mAb-goat-α-mouse-HRP in 1% PBSA-1%NGoS and substrate was added (0.05M NaOH pH5.0, AEC in dimethylformamide, H_2O_2) until staining appeared.

Picrosirius red staining

Quantification of collagen content was performed according to Perrée et al. with minor modifications [22], using picrosirius red staining and digital image microscopy with circulary polarized light. The section image was converted into a greyvalue image and regions of interest (ROI) were drawn to select the three different arterial layers. The collagen content is linearly proportional to the gray value as assessed by the hydroxyproline assay (Hypronosticon kit, Organon Teknika, Oss, The Netherlands). The collagen content of each layer is presented as the ratio of the col-

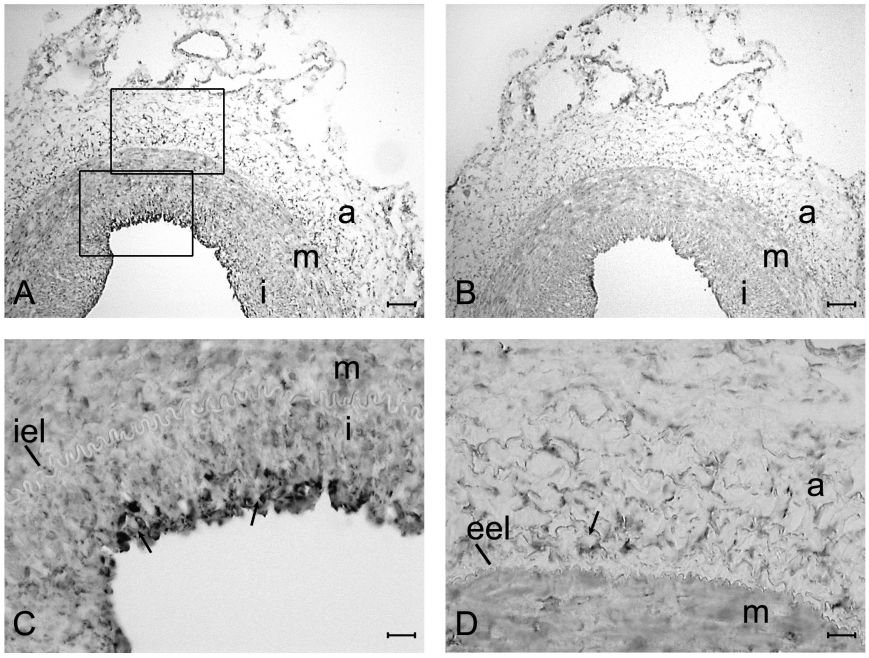

Figure 3: *Localization of Hsp47 (in blue) in arterial cross section 14 days after balloon dilation. A:. Localization of Hsp47 with a magnification of intimal layer (bottom frame) and of the adventitia (top frame) (bar= 500 μm). B: Isotype staining (bar= 500 μm). C: Localization of Hsp47 in intimal layer (bar= 125 μm), D: and adventitial layer (bar= 125 μm). (a= adventitia, m= media, i= intima, IEL= internal elastic lamina, EEL=external elastic lamina, arrows indicate positive cells for Hsp47).*

lagen content of the operated layer compared to the collagen content of the adventitial layer of the contralateral artery. This to clarify the relative distribution of collagen fibers through the artery.

Statistical analysis

Statistical analysis of the data was performed using a Wilcoxon matched pairs signed rank sum test. Data are presented as ratio operated versus control mean ± the standard error of the mean. P values of < 0.05 were considered statistically significant.

RESULTS

Structural arterial changes and intimal hyperplasia after balloon injury

Two days after balloon dilation, the average arterial internal elastic laminae area (IEL) increased (+3.5%) compared to post-dilation. From day 7 we observed a decrease in IEL, corrected for neointima formation, which was significant at day 14

(-17.7%, p=0.05). At day 28, the IEL had decreased by -25.5% (p=0.01)(Figure 1). This arterial shrinkage was accompanied by the onset of neointima formation at day 7 (1.5%) and increased in time (16.7% at day 28) (Figure 1). No neointima was detected in contralateral uninjured arteries. Only a few macrophages were detected in the artery at all time points (not shown).

Hsp47 and procollagen I expression levels after balloon injury

Quantitative RT-PCR showed that after balloon dilation Hsp47 mRNA levels increased at day 2 (p=0.25) (Figure 2A), reached significance at day 7 (p=0.008) and day 14 (p=0.002), and declined at day 28. Western blotting (Figure 2B) showed that the increase in mRNA was accompanied by an increase of Hsp47 protein levels at all time-points with a maximum at day 14 (p=0.001).

Hsp47 protein (in blue) was localized in the neointima (Figure 3A+C), the adventitial layer (Figure 3A+D) and some staining was found in the media (Figure 3A). No staining was found using the isotype control (Figure 3B). Cells positive for Hsp47, in both the intima and adventitia, stained positive for vimentin, thus being fibroblast-like cells (data not shown).

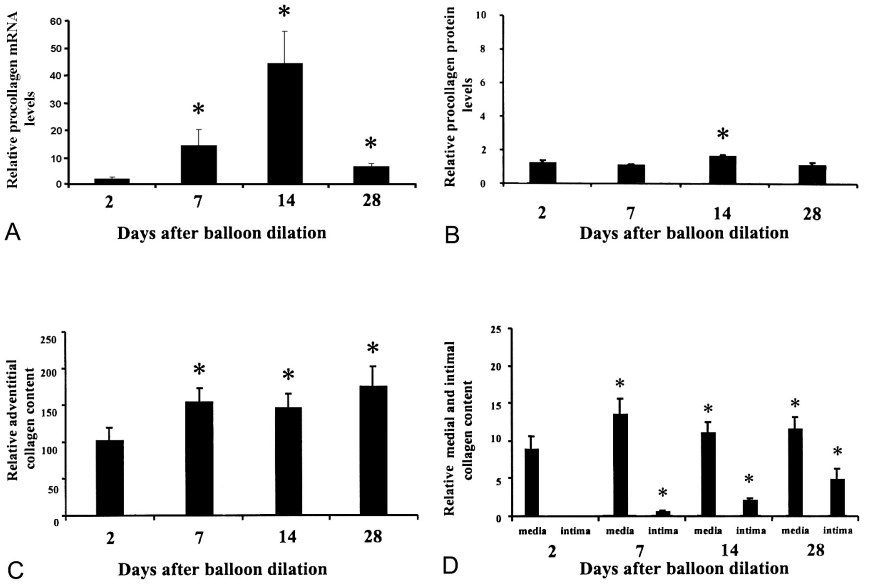

Figure 4: *Collagen expression patterns and collagen content 2, 7, 14 and 28 days after balloon dilation. Procollagen I mRNA (A) and free procollagen I protein (B) expression of balloon dilated arteries compared to contralateral control arteries. (C,D) Collagen content of adventitia, media and intima in balloon dilated arteries compared to the adventitia of contralateral control arteries at each timepoint. (Data are presented as the ratio operated / control mean ± sem; femoral and iliac arteries of each survival time are pooled. N=6-8 rabbits per time point, * = P<0.05).*

Together with the increase in Hsp47 expression, an increase in collagen-I mRNA levels was found at day 7 (p=0.001) with a maximum at 14 days (p=0.001; Figure 4A). Despite the increase in Hsp47 protein and collagen I mRNA, there were no differences in the total protein levels of procollagen type I at day 2, 7 and 28. Only at day 14 there was a small but significant increase (1.6 fold, p=0.004, Figure 4B) compared to control arteries.

Collagen fiber content after balloon injury

The amount of collagen content in the adventitia showed an increase at day 7 (155%, p=0.007) compared to the contralateral uninjured arteries. The increased adventitial collagen content remained constant at day 14 and 28 (Figure 4C). Also in the media a significant increase in collagen content at day 7 (p=0.013; Figure 4D) was found compared to the adventital collagen content of contralateral uninjured arteries. Similar to the adventitia, medial collagen content didn't increase further at 14 and 28 days. The amount of collagen content in the intima (Figure 4D) is relatively low, compared to the adventitia, and increased from 0.5±0.1% at day 7 to 5.8±1.7% at day 28.

Levels of procollagen I bound to Hsp47 after balloon injury

To investigate the discrepancy between increased collagen synthesis, reflected by increased levels of collagen I mRNA, Hsp47 mRNA, Hsp47 protein levels and increased collagen content on one site and the absence of increased procollagen I protein levels on the other site, we measured the levels of procollagen I bound to Hsp47. For this, Western blots of total protein lysates of the balloon dilated arteries were incubated with the mAb against Hsp47 and showed a protein complex containing Hsp47 and procollagen bound to Hsp47 (Figure 5A, †). [21, 23] We confirmed that this complex contained procollagen by incubating one half of the Western blot with the mAb against Hsp47 and the other half with the pAb against collagen I. Comparing these blots, the bands detected by Hsp47 mAb (Figure 5A, lane 1) and by collagen I pAb (Figure 5A, lane 3) had the same molecular weight. As expected, in the collagenase treated samples the procollagen bands were degraded (Figure 5A, lane 2 and 4). Determination of Hsp47 bound procollagen I levels with the Hsp47 antibody at the different time-points after balloon dilation showed a similar expression pattern as Hsp47 protein with a maximum at day 14 (p=0.001) compared to contralateral control arteries (Figure 5B).

Crosslinking of Hsp47 and procollagen in v-SMC with DSP before the protein isolation preserves the binding between Hsp47 and procollagen during isolation. This resulted in an increased signal of the Hsp47-procollagen complex (Figure 5C, lane 1+2, †).

To confirm the results of increased Hsp47 expression and increased procollagen I bound to Hsp47, but constant free procollagen I levels found *in vivo*, we repeated the experiments ex vivo with aortic rings. *Ex vivo* experiments revealed increased Hsp47 levels after injury (Figure 5C, lane 4, Δ) and even more Hsp47 expression after injury and TGF-β1 stimulation (Figure 5C, lane 5, Δ) compared to uninjured control aortic rings (Figure 5C, lane 3, Δ). This was accompanied by increasing levels of procolla-

gen I bound to Hsp47 (Figure 5C, bottom panel, †) but not by the free procollagen I (Figure 5C, top panel, ††), confirming the observed results in vivo.

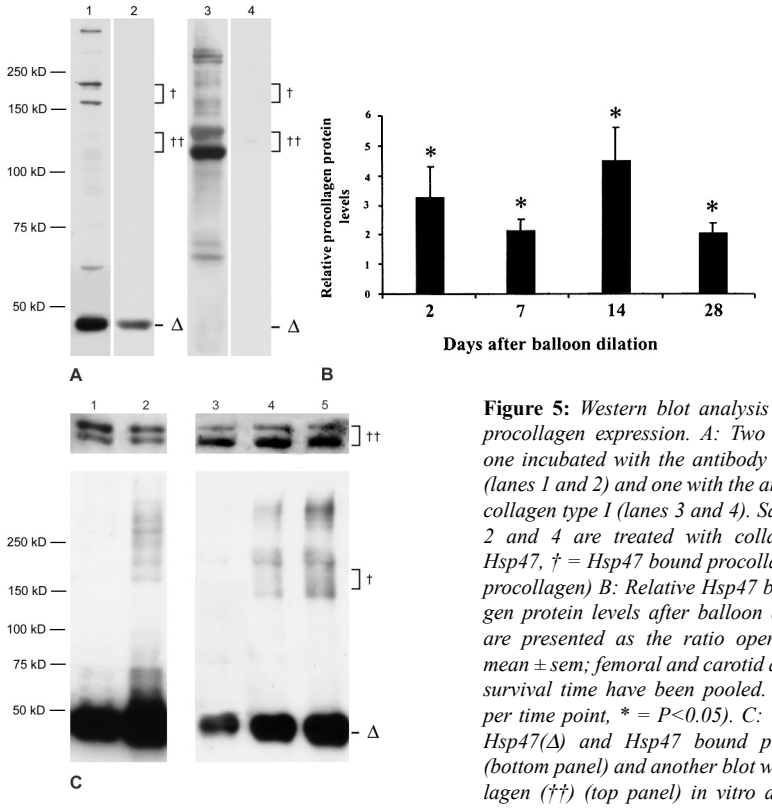

Figure 5: *Western blot analysis of Hsp47 and procollagen expression. A: Two Western blots; one incubated with the antibody against Hsp47 (lanes 1 and 2) and one with the antibody against collagen type I (lanes 3 and 4). Samples in lanes 2 and 4 are treated with collagenase. (Δ = Hsp47, † = Hsp47 bound procollagen, †† = free procollagen) B: Relative Hsp47 bound procollagen protein levels after balloon dilation. (Data are presented as the ratio operated / control mean ± sem; femoral and carotid arteries at each survival time have been pooled. N=6-8 rabbits per time point, * = P<0.05). C: Western blot of Hsp47(Δ) and Hsp47 bound procollagen (†) (bottom panel) and another blot with free procollagen (††) (top panel) in vitro and ex vivo. In vitro: Levels in v-SMC without- (lane 1) and with cross-linking (lane 2). Ex vivo: Levels after injury (lane 4) or after injury and TGF-β1 stimulation (lane 5) compared to control (lane 3).*

MMP-1, MMP–2 and Moesin levels after balloon dilation

To study collagen degradation next to collagen synthesis and collagen deposition we used zymography. In our model, MMP-1 levels are significantly increased in the balloon dilated arteries after seven days (p=0.01) and reaching a maximum at 14 days (p=0.003) (figure 6A). Active MMP-2 levels are increased compared to control levels at two days (p=0.004) after balloon dilation and reached its maximum also at 14 days (p=0.002) (figure 6B).

To explore cell migration, we studied Moesin protein levels which is a marker for SMC migration.[24] We found that Moesin levels started to increase at day 7 (p=0.07) and reached significance at day 14 (p=0.04). (figure 6C).

DISCUSSION

Mechanisms of arterial remodeling and neointima formation as a response to injury are still unclear. Until now, *in vivo* studies have focussed on the degradation of the extracellular matrix, in particular collagen, and little attention was given to collagen synthesis and collagen fiber content in relation to collagen degradation in time.
After balloon dilation, luminal narrowing is the result of 2 processes: arterial shrinkage and intimal hyperplasia (Figure 1).[3,25,26] In our model, intimal hyperplasia was observed from day 7, increasing in size at 14 and 28 days and was in accordance with previous reports by Rasmussen et al and Barron et al.[27,28] Also the arterial shrinkage, from day 14 and progressing in time, was observed in previous studies confirming the reproducibility of the model we used. [3,29,30]

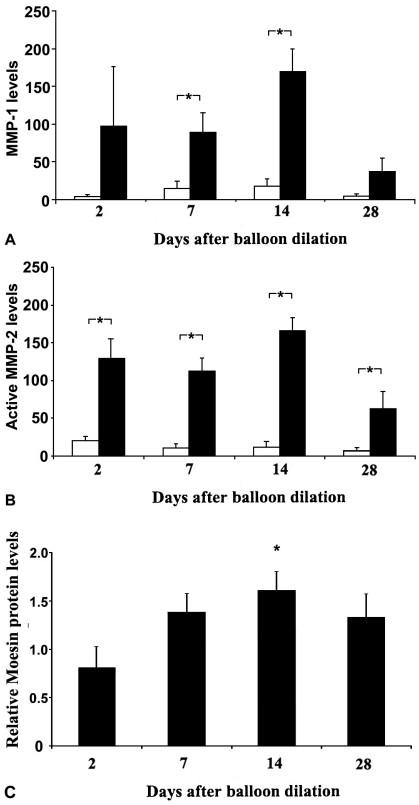

Figure 6: *MMP-1, MMP-2 and Moesin expression levels after balloon dilation. (A) Relative MMP-1 expression levels after balloon dilation compared to control. (B) Relative active MMP-2 levels after balloon dilation compared to control levels. (White bars = control arteries, black bars = balloon dilated arteries; data are presented as the measured mean number of pixels ± sem). (C) Relative Moesin protein levels after balloon dilation compared to control levels (data are presented as the mean ratio balloon dilated / control arteries ± sem) (N=6-8 rabbits per time point, * = p<0.05).*

After balloon dilation we observed increased collagen mRNA levels, accompanied by increased Hsp47 mRNA and protein levels with a maximum increase at 14 days (Figure 2A,B + Figure 4A). Karim and colleagues showed an increase in collagen I mRNA at day 7 which was two to three times the control levels and returned to basal levels at 4 weeks, but missing time points in-between.[31] We confirmed these data but

found the maximum increase in collagen mRNA levels at 14 days. Using intracellular Hsp47 localization, we showed that collagen synthesis was mainly observed in fibroblasts of the adventitial and intimal layer. This observation is accordance with Shi et al [32,33] who showed an increase in procollagen levels in the adventitia and intima after balloon dilation of porcine coronary arteries and illustrated the contribution of adventitial fibroblasts to neointima formation after arterial injury. However, Murakami et al described no adventitial Hsp47 expression after arterial injury of the rat.[34] A possible explanation is that in this model only the endothelium is damaged and no or less arterial shrinkage might occur.

One explanation for the peak in collagen mRNA levels at 14 days could be a maximal collagen fiber formation. We found increased collagen content present within the adventitial, medial and intimal layers at day 7, 14 and 28 after balloon dilation (Figure 4C+D). However, a maximal increase in collagen content was reached at day 7 in the media and adventitia, and the relative fiber content of the intima is low. Therefore, we think that the peak in collagen mRNA levels found at 14 days cannot be explained by collagen fiber formation alone.

Another explanation can be deduced from the matrix metalloproteinases MMP-2 and MMP-1 levels (Figure 6) which were maximal at 14 days compared to control arteries. Therefore, we hypothesized that the peak in collagen and Hsp47 levels at 14 days is needed for cell migration [19] and fine tuning, or reshaping of the collagen fibers in the arterial wall, essential in the process of arterial restructuring. In these processes, the MMPs are essential as they are the only enzymes capable of degrading collagen and are essential for cell migration.[20] For remodeling, our data show that the maximal arterial shrinkage is between day 7 and 14 (figure 1) when collagen mRNA levels are maximal. This association suggests a role for collagen turn-over in remodeling.

Blindt et al [24] described SMC migration as an effect of Moesin up-regulation. Moesin expression was studied to explore the time-course of SMC migration. We observed an increase of Moesin levels starting after 1 week and reaching a significant maximum level at day 14 (figure 6C), implicating that SMC migration indeed occurs after the first week following injury.

Based upon the well described co-expression of Hsp47 and procollagen I [15-17], we expected a similar time course of procollagen I and Hsp47 protein expression after the experimental procedures. We found that levels of Hsp47 (figure 2B) and Hsp47-bound procollagen I [21,23], (Figure 5B) increase in time. Usually crosslinking and immunoprecipitation are used to study protein-protein interactions, like Hsp47-bound procollagen. We found the complex present *in vivo* after the isolations without crosslinking and confirmed these results *ex vivo*. *In vitro* crosslinking of v-SMC, before protein isolation, preserves the binding of procollagen I to Hsp47 (Figure 5C, lane 1+2) and increases the signal of the complex. This implicates that the increases found of procollagen bound to Hsp47 *in vivo*, without crosslinking, will be underestimated (Figure 5B). Although Hsp47 and Hsp47-bound procollagen levels increased, we found only a small increase in procollagen I levels not bound to Hsp47, at day 14 and not at day 7 (Figure 4B).

Hypothetically, these results suggest that arterial adaptation to injury initiates the following collagen synthesis pathway (Figure 7): In the normal control situation there is a constant throughput (small arrow) of procollagen with normal (N) levels intra- and extracellular. After arterial injury there is an increase in procollagen synthesis, reflected by Hsp47 bound procollagen. This results in an increase in extracellular collagen fiber content and/or procollagen degradation. However, the free procollagen levels remain constant, suggesting a higher throughput of free procollagen (large arrows) after injury. This balance, between collagen synthesis and collagen fiber content and/or procollagen degradation might be disrupted when the increase is collagen synthesis is too large (Figure 4B, day 14).

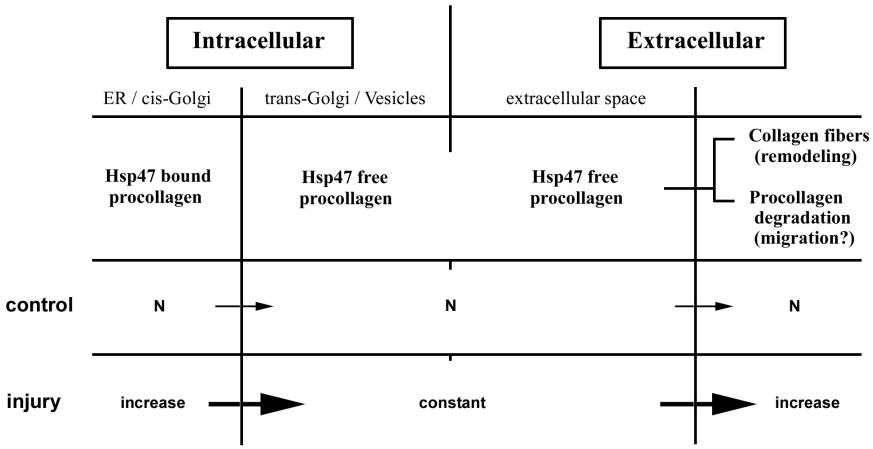

Figure 7: *Hypothetical pathway of Hsp47 and procollagen I turnover in the arterial adaptation to injury. In the normal control situation there is a constant throughput (small arrow) of procollagen with normal (N) levels intra- and extracellular. After arterial injury there is an increase in procollagen synthesis, reflected by Hsp47 bound procollagen. This results in an increase in extracellular collagen fiber content and/or procollagen degradation. However, the free procollagen levels remain constant, suggesting a higher throughput of free procollagen (large arrows) after arterial injury.*

Strauss et al [6,7] studied collagen synthesis and degradation in a double injury model, in which they found a relative delay in collagen accumulation in the artery. In this model they did not study arterial remodeling, and although the double injury model is more human like, there is already a response to injury present after the first injury which makes it difficult to study the initial arterial response to injury. They also excluded the adventitial layer, in which the bulk of collagen is present. The delay in collagen accumulation described was found after the second injury, but there is also an increased collagen content present directly after the second injury compared to the non-dilated control artery. This is probably due to the first injury and is accumulated in the three weeks between the operations. They also found that significant amounts

of newly synthesized collagen did not accumulate in the vessel wall which is consistent with our results that newly synthesized collagen is not only used for collagen deposition after the first week.
Thus, the present results support the data of Strauss et al.[6,7] and Karim et al.[31] In addition to them we analyzed the process at different expression levels; procollagen mRNA, procollagen protein, collagen content and MMP activity. We associated these levels with the morphometrical changes of the arteries and included the adventitial layer in our analysis to better understand the mechanisms that play a role in the arterial response to injury. Furthermore, we suggest a role for de novo synthesis and degradation of collagen in cell migration.
In conclusion, we report that in the first week after arterial injury increased collagen content is associated with collagen synthesis and breakdown and Hsp47 expression. However, after 1 week collagen turnover increased further in contrast to collagen content. We did not demonstrate a direct relation between migration, collagen synthesis and collagen degradation. However, the association between increased levels of collagen turnover and moesin expression, suggests that these increases found after the first week are probably needed for reshaping or remodeling the artery and for cell migration, pointing to a new additional role of collagen turnover in the arterial response to injury.

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5 Involvement of the proprotein convertase furin in the arterial response to injury

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ABSTRACT

Background: *The proprotein convertase furin is a proteolytic activator of proproteins, like membrane type 1-matrix metalloproteinase (MT1-MMP) and transforming growth factor β (TGF-β), that are described in the arterial response to injury. However, the involvement of furin in the arterial response to injury has not been studied yet. We studied furin expression and the effect of a specific furin inhibitor, á1-antitrypsin Portland (α1-PDX), on arterial injury following balloon dilation. We also investigated the expression and signaling pathways of MT1-MMP and TGF-β, after arterial injury and explored the effect of furin inhibition on their activation.*

Methods and Results: *NZW Rabbit femoral and iliac arteries (N=42) were balloon dilated unilaterally and harvested after 2, 7, 14, 28 or 42 days. Furin mRNA levels were increased after 2 and 7 days. MMP-2 and MT1-MMP levels were increased after day 7 and TGF-β signaling, by phosphorylating Smad 1/5 and 2/3, was increased at all time points. Ex vivo inhibition of furin, by adenoviral over-expression of α1-PDX in aortic rings, blocked proTGF-β activation and Smad phosphorylation, and reduced MT1-MMP and MMP-2 activation (N=3). In vivo adventitial inhibition of furin (N=9) resulted in a reduction of 13.1 +/- 5.2% in advential and 23.6+/-7.9% in intimal areas (P<0.05), but had no effect on lumen size due to decreased vessel areas.*

Conclusions: *This study demonstrates that furin is involved in the arterial response to injury possibly through inhibition of the TGF-β -Smad signaling pathway and identifies furin as a possible target to inhibit intimal hyperplasia.*

SUBMITTED FOR PUBLICATION

INTRODUCTION

Furin, a member of the proprotein convertases, is a calcium dependent protease and functions mainly as a proteolytic activator of proproteins.[1] The potential role of furin in pathogenesis of disease[2] has lead to the development of specific inhibitors. A potent specific furin inhibitor is a mutant form of α1-antitrypsin, the α1-antitrypsin Portland (α1-PDX). It contains in its reactive site the minimal consensus sequence for efficient processing by furin, and is more than 3000-fold more effective than α1-antitrypsin at inhibiting furin in vivo, moreover, it does not inhibit either elastase or thrombin.[3]

Increased furin expression has been reported in flow-induced arterial remodeling.[4] However, it is not known if furin is involved in the arterial response to injury, characterized by neointimal formation and arterial remodeling. In addition, several substrates that are activated by furin have been described in the arterial response to injury, like pro-membrane type 1- matrix metalloproteinase precursor (proMT1-MMP),[5] and the pro-transforming growth factor β (proTGF-β).[6] Although these furin substrates are described to be present in the artery after injury, it is still unclear when proMT1-MMP is activated and if the activated TGF-β is able to induce downstream signaling pathways through phosphorylation of Smad 1/5 and Smad 2/3. It is also unknown if furin is involved in the arterial response to injury and if furin inhibition modulates neointima formation and/or arterial remodeling.

We investigated furin expression, the MT1-MMP – MMP2 activation pathway and TGF-β – Smad signalling pathway in time following balloon dilation. This revealed that furin, MT1-MMP, MMP-2 and phosphorylated Smad 1/5 and 2/3 levels increased after balloon dilation. We locally administered an adenovirus, expressing the potent furin inhibitor α1-PDX, on the adventitia of the balloon dilated segment. Inhibition of furin after balloon dilation resulted in a reduction in advential and intimal areas. Our study demonstrates involvement of furin in the arterial response to injury and suggests that furin is a potential interesting target to intervene in neointima formation.

MATERIALS AND METHODS

Animals

Animals were housed conform to the Guide for the care and Use of Laboratory Animals (NIH publication No.85-23, 1985) and all experiments were approved by the ethical committee on animal experiments of the University Medical Center, Utrecht. Forty-two New Zealand White rabbits (Broekman Charles River, 3-3.5 kg) were anesthetized by methadone (0.15 ml) and vetranquil (0.15 ml) followed by etomidate (1 mg/kg) and ventilation with N2O:O2/0.6% halothane.

Femoral and external iliac arteries of thirty-five rabbits were balloon dilated as described before.[7] Arterial lumen diameter was determined using angiography after balloon dilation and at termination. Geometrical remodeling (RM) and intimal for-

mation (IH) were analyzed as previously described.[7] The balloon dilated segments and the contralateral control arteries were harvested after 2, 7, 14, 28 and 42 days and immediately frozen at -80°C for RNA and protein isolation[7] (N=6-7 rabbits per time point).

Quantitative RT-PCR

Rabbit furin (Forward: 5'- ccatccaggctggttttgta -3'; Reverse: 5'- gtccattaaatagaaccaacaatgc -3') and ribosomal 18S (Forward:5'-tcaacacgggaaacctcac-3'; Reverse:5'-acaaatcgctccagcaac-3') primers were designed using the Prime program at CMBI (Nijmegen).

Quantitative RT-PCR was performed as previously descibed.7 The PCR reactions started with 2 min at 94°°C followed by 40 cycles of: 30 sec at 94°C, 30 sec at 60°C and 60 sec at 72°C. Data are presented as the ratio of the dilated and control arteries.

Survival	RM (mm)	sem	p-value	IH (mm2)	sem	p-value
2	0.1	0.07	0.17	0		
7	-0.04	0.09	0.69	0.096	0.304	0.3
14	-0.27	0.12	0.05	0.049	0.040	0.04
28	-0.43	0.11	0.01	0.305	0.003	0.003
42	-0.59	0.07	<0,001	0.262	0.017	0.018

Table 1: *Remodeling (RM) of the artery and intimal hyperplasia (IH) 2, 7, 14, 28 and 42 days after balloon dilation. RM is calculated as mean relative change +/- sem (mm) in internal elastic laminae (IEL) diameter between post dilation and at termination. IH is presented as mean absolute area (mm^2) +/- sem ($p<0.05$ = significant).*

Western blotting and Zymography

For Western blotting, samples (12 µg) were separated on 10% SDS-PAGE gel and transferred onto a Hybond-ECL membrane (Amersham). MT1-MMP was detected with a monoclonal antibody for MT1-MMP (clone113-5B7, Oncogene) and a goat-α-mouse-HRP (DAKO). Phosphorylated Smad 1/5 and Smad 2/3 with a polyclonal antibody for pSmad 1/5 (clone sc-12353, Santa Cruz) or for pSmad 2/3 (clone sc-11769, Santa Cruz) and a rabbit-α-goat-biotin (DAKO) and streptavidin-HRP, followed by chemiluminescence substrate (Sigma) and exposed to the ChemiDoc XRS system (Biorad). Negative controls were performed using an isotype control antibody and by omitting the first antibody.

Zymography was performed as described before.[7] The number of pixels measured by western blotting and zymography are presented as the ratio between dilated and contralateral control arteries.

α1-PDX adenoviral construct

The α1-PDX construct, containing the furin inhibitor (modified α1-antitrypsin), was kindly provided by Dr. G. Thomas (Portland, USA). The α1-PDX was cloned into the Ad-Easy Xl vector (Stratagene) (Ad.CMV.PDX), according the manufacturers

protocol. We also created two control viruses, an empty virus (Ad.CMV.Empty) and a virus expressing β-galactosidase (Ad.CMV.LacZ). Cesium Chloride purification was performed on amplificated virus stocks and titers (plaque forming units (pfu)/ml) were determined by repeated plaque assays.[8]

Ex vivo adventitial gene delivery and protein expression

Normal rabbit aortas (N=3) were harvested and transfected peri-adventitially, with the PDX construct or the empty virus (50µl of 2.10^{10} pfu/ml), by slight pressing with a brush.[9] The segments were cut into aortic rings for culture (3+7 days) as described before[7] and total protein was isolated. Western blotting was performed for MT1-

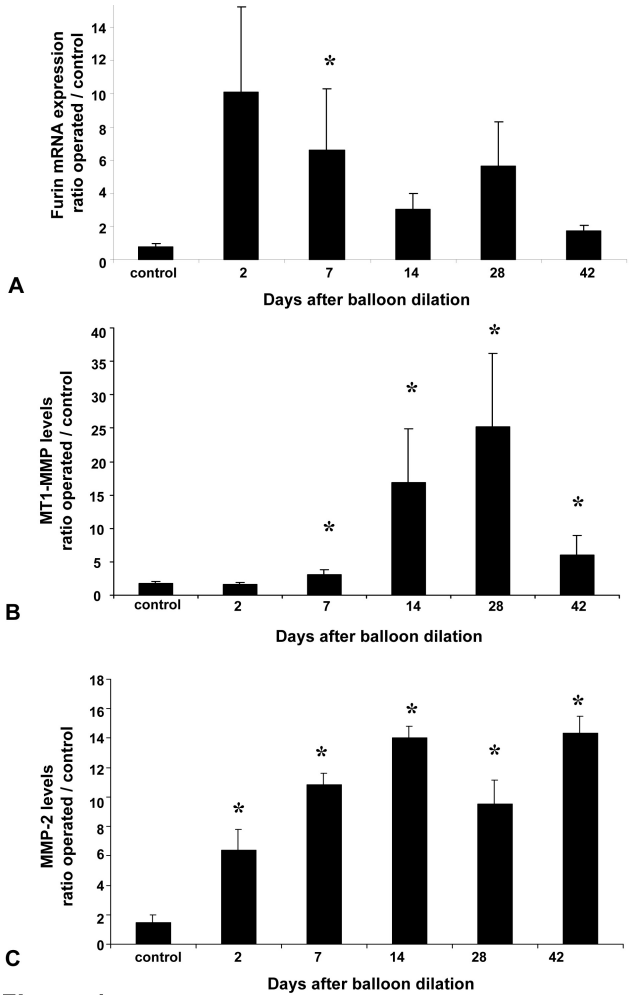


Figure 1: *(A) Furin mRNA expression 2, 7, 14, 28 and 42 days after balloon dilation / contralateral control artery. Furin expression is significant increased at day 2 and 7. Expression of activated membrane type 1- matrix metalloproteinase (MT1-MMP) (B), and activated MMP-2 (C) was calculated relative to levels detected from contralateral control arteries. Injury increases levels of activated MT1-MMP and MMP-2 protein levels. (ratio mean number of pixels ± sem) (* = p<0.05).*

MMP and for phosphorylated Smad, and for TGF-β by using rabbit-α-TGF-β (R&D systems) and goat-α-rabbit-HRP (DAKO) antibodies. Zymography was performed as described above.

In vivo Adventitial gene delivery

The Ad.CMV.LacZ virus was applied on the artery to explore transfection efficiency of the used peri-adventitial brushing method. β-galactosidase expression was stained as described by the manufacturer of the Ad-Easy Xl vector.

Both femoral arteries of rabbits (N=11) were carefully explored and embedded in a saline solution containing 5 mg/ml papaverin. Balloon dilation was performed bilateral as described before[7] and, either the Ad.CMV.PDX virus or an empty virus (Ad.CMV.Empty) was applied locally (50µl of 2.10^{10} pfu/ml) on the 2 cm long dilated arterial segment. Each rabbit received the PDX construct at one side and the control construct at the contralateral artery. For applying the virus peri-adventitially, the arteries were lifted and the viral constructs were spread by slight pressing with a brush as described before.[9] After a minute the arteries were put back in position and tissues were closed. Angiographic images were taken before, during, and after balloon dilation and at termination and arterial diameters were digitally analyzed. Rabbits completely recovered after surgery and were terminated after 1 (N=1), 3 (N=1), and 14 days (N=9).

Histological analysis

Transfected balloon dilated arteries (N=9) were harvested after 14 days fixated in 4% formaldehyde and embedded in paraffin. Adventital, medial, intimal and lumen areas were analyzed by cross sectional analyis (10 sections per artery, 500µm in between) on EvG stained sections and median values were compared. Regions of interest were drawn on digital images, and computerized analysis was performed to analyze the different areas (Analysis 3.2). Regions of interest (ROI) were drawn at the outside of the adventitia (1), at the EEL (2) and IEL (3), and the luminal border (4) (Figure 5). The adventitial area is defined as the area between ROI 1 and 2, the medial area between ROI 2 and 3, and the intimal area between ROI 3 and 4.

Statistical analysis

Statistical analysis of the data was performed using a Wilcoxon matched pairs signed rank sum test. Expression data are presented as ratio operated versus control mean ± the standard error of the mean. The α-PDX and empty virus treated arteries were compared by the median of each artery. P values of < 0.05 were considered statistical significant.

RESULTS

Remodeling and neointima formation after balloon injury

A decrease in IEL was assessed from day 7, which became statistically significant at day 14 (-0.27 mm, p=0.05) (Table 1). At day 28, the IEL had decreased by –0.43 mm

(p=0.01) and at day 42 by -0.59 mm (p<0.001). The onset of neointima formation was also detectable at day 7 (0.096 mm2 increase in intimal area) and this increased till day 28 (0.305 mm2), when it reached a plateau till day 42 (Table 1). As expected no neointima was detected in contralateral uninjured arteries.

Arterial furin, MT1-MMP and MMP-2 levels after injury

After balloon dilation, we found increased furin mRNA levels at 2 and 7 days (p=0.01 and p=0.004, respectively)(Fig 1A) compared to contralateral control arteries. After 2 days, furin mRNA levels declined in time and returned to basal levels. Active MT1-MMP protein levels were significantly increased at day 7 (p=0.003), reached a maximum at 28 days (p=0.038), but remained elevated until 42 days (p=0.016)(Fig 1B). Active MMP-2 levels were increased after balloon dilation com-

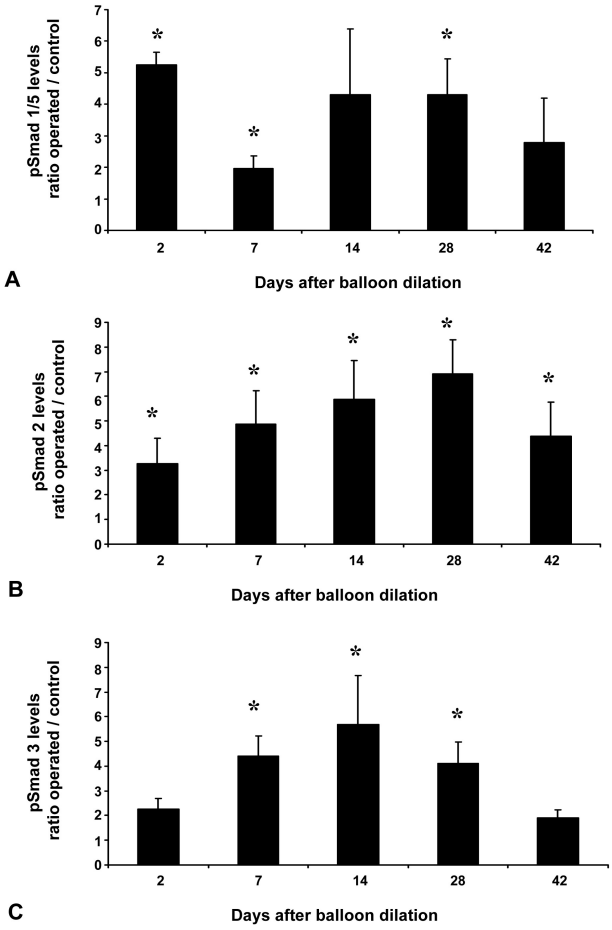


Figure 2: *Western blot analysis of phosphorylated Smad 1/5 (A: pSmad 1/5), phosphorylated Smad 2 (B: pSmad 2), and phosphorylated Smad 3 (C: pSmad 3). Expression levels were calculated relative to levels from contralateral control arteries. pSmad expression levels are increased in time after balloon dilation. (data are presented as the measured mean number of pixels ± sem) (* = p<0.05).*

pared to control levels at 2 days (p=0.004) and also reached its maximum at 14 days (p=0.002). The active MMP-2 levels remained elevated throughout the period studied (Fig 1C).

Arterial Phosphorylated Smad 1/5 and 2/3 levels after balloon injury
After balloon dilation, we observed an increase in phosphorylated Smad 1/5 (pSmad 1/5) at day 2, 7, and 28 (p=0.03, p=0.04, p=0.009)(Fig 2A).
Phosphorylated Smad 2 (pSmad 2, Fig 2B) was also increased from day 2 onwards (p=0.009) and slowly reached a maximum at day 28 (p=0.009). The same pattern was seen with pSmad 3, but with a maximum at day 14 (p=0.008) (Fig 2C).

Ex vivo furin inhibition and MT1-MMP, MMP-2, and TGF-β1 levels.
We transfected aortic rings ex-vivo to study the effect of furin inhibition on MT1-MMP and TGF-β activation. α1-Antitrypsin is expressed 3 and 7 days after α1-PDX transfection, and is not present in the control aortic rings (Fig 3A). MT1-MMP activation was inhibited after 3 days of culture while a reduction in MT1-MMP activation and MMP-2 activation was found 7 days after transfection with Ad.CMV.PDX (Fig 3B+C, respectively). Interestingly, 3 and 7 days after transfection with Ad.CMV.PDX, we found no TGF-β activation (Fig 3D) in contrast to the aortic rings

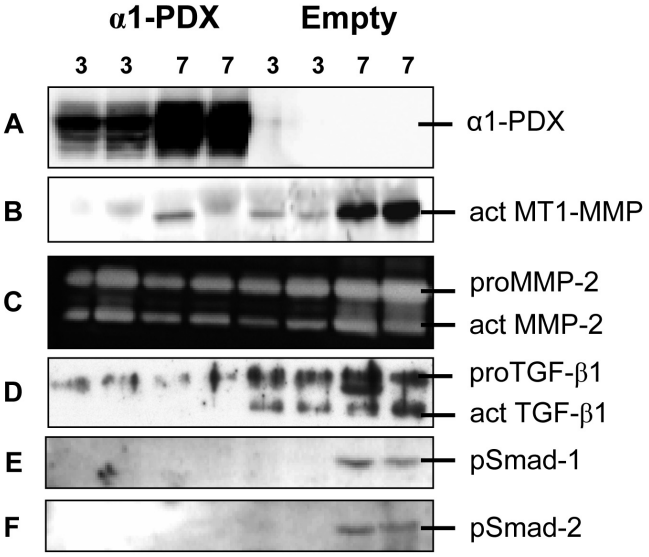


Figure 3: *Western blot and zymography analysis of α1-antitrypsin (α1-PDX) (A), MT1-MMP (B), MMP-2 (C), transforming growth factor β (TGF-β) (D), phosphorylated Smad-1 (pSmad-1) (E), and phosphorylated Smad-2 (pSmad-2)(F) expression levels. Aortic rings were cultured 3 and 7 days after transfection with Ad.CMV.α1-PDX or Ad.CMV.Empty. The expression of the modified α1-antitrypsin (α1-PDX) reduced MT1-MMP and MMP-2 activity, and inhibited active TGF-β levels and phosphorylation of Smad-1 and 2.*

transfected with Ad.CMV.Empty. TGF-β downstream signaling was also affected, while both pSmad 1 and pSmad 2 are present 7 days after culture in the empty virus infected aorta rings, no phosphorylated Smad could be detected in the PDX infected rings (Fig 3E+F).

Angiographic and morphometric analysis after á1-PDX transfection

Transfection efficiency in vivo was tested by locally administering a β-galactosidase producing adenovirus. The peri-adventitial gene delivery was able to transduce the adventitial layer but did not transfect medial smooth muscle cells (SMCs)(Fig 4a). The α1-PDX expression could be detected by Western blotting in vivo, 1 and 3 days after transfection (Fig 4b).

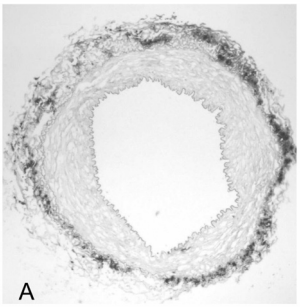
Nine rabbits were balloon dilated in both femoral arteries and transfected with Ad.CMV.PDX or Ad.CMV.Empty. The angiographic diameters measured before dilation, during balloon dilation and post-dilation, did not differ between the Ad.CMV.PDX and Ad.CMV.Empty transduced arteries. Also at termination no significant differences were found in lumen diameter, and in late lumen loss (LLL= diameter post-dilation – diameter termination) (table 2).

Morphometry confirmed the angiographic data and revealed no difference in mean luminal area between the PDX and control treated arteries (PDX: 0.43±0.07 mm^2 vs Empty: 0.44±0.06 mm^2 α1-antitrypsin) (table 2). Reductions in the outside of the adventitia, EEL, and IEL, were observed, but were not significant (Table 2). The medial areas (PDX: 0.42±0.03 mm^2 vs Empty: 0.42±0.03 mm^2) did also not differ significantly. Comparison of the adventitial and intimal areas, however, revealed a 13.1 +/- 5.2% reduction in adventitial area (PDX: 0.44±0.02mm^2 vs Empty: 0.53±0.04 mm^2, p=0.03) and a 23.6 +/- 7.9% reduction in intimal area (PDX: 0.40±0.05mm^2 vs Empty: 0.49±0.02mm^2, p=0.05) in the PDX treated arteries. (Fig 5).

DISCUSSION

Furin is a proteolytic activator of proproteins. Several possible substrates have been described in the arterial wall after injury, including MT1-MMP and TGF-β. However, the role of furin after arterial injury and subsequent activation of MT1-MMP and TGF-β has not been explored. We found increased mRNA expression of furin at 2 and 7 days after balloon injury in rabbits. However, unfortunately all antibodies tested for furin were not able to detect rabbit specific furin neither by Western blotting nor by immunohistochemistry. This increase in furin expression was accompanied by the onset of arterial shrinkage.

Both the MT1-MMP – MMP-2 activation pathway and the TGF-β signaling pathways were activated after balloon injury. Active MT1-MMP levels were increased from day 7, with a maximum at 14 days and active MMP-2 levels increased at day 2, with also a maximum at 14 days, confirming previous reports on MMP-2 expression.[10,11] Analyzing the TGF-β signaling cascade showed that both pSmad 1/5 and pSmad 2/3 were increased from day 2 following balloon dilation, suggesting that



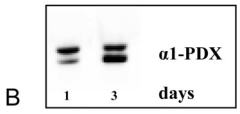


Figure 4: *(A) Staining for β-galactosidase 3 days after peri-adventitial gene delivery using 1.10^9 pfu Ad.CMV.lacZ was able to transfect the adventitial area. (B) By Western blotting α1-antitrypsin, produced by α1-PDX, could be detected at day 1 and 3.*

TGF-β is able to regulate downstream targets. This is in agreement with previous reports showing that active TGF-β1 levels are increased between 2 hours and 7 days, with a maximum at 3 days,[12] after PTCA injury in porcine arteries. Early after injury, TGF-β1 expression was localized within the adventitia and from day 7 also in the developing neointima,[13,14] suggesting that an adventitial adenoviral approach to inhibit TGF-β1 signaling might be effective.

To test if adenoviral α1-PDX was able to inhibit furin-induced MT1-MMP and TGF-β activation we transfected aortic rings ex-vivo with α1-PDX or the control virus. We observed a reduction in both MT1-MMP activation and MMP-2 activity. Activation of TGF-β and subsequent phosphorylation of Smad 1 and 2 was also inhibited.

Subsequently, we balloon dilated femoral arteries in vivo and inhibited furin by adventitial expression of α1-PDX. Fourteen days after balloon dilation, we observed no differences in arterial lumen sizes and in late lumen loss between the PDX treated arteries and the control arteries. We observed a significant reduction, however, in intimal and adventitial areas in the PDX treated group, showing that furin is involved in the arterial response to injury.

Our in vivo and ex vivo data suggest that one function of furin in the arterial response to injury is cleaving of pro-TGF-β thereby increasing TGF-β levels in the arterial wall. We observed an early increase in furin expression after arterial injury, corresponding with previously observed increases in TGF-β1 expression and activation. Moreover, TGF-β signaling pathways were increased early after injury and the peri-adventitial inhibition of furin resulted in reduced TGF-β levels as well as reduced TGF-β signaling via Smad phosphorylation. MT1-MMP activation occurred later in time after balloon dilation and is therefore less susceptible for inhibition by an adenoviral approach. Also the coordinated increase in expression of furin and TGF-β1 after increased shear stress[15] suggests their relation. Moreover, in furin knockout cells it was shown that TGF-β maturation was absent, while active MT1-MMP was still present, suggesting the existence of a furin-independent activation of MT1-MMP.[16]

The reduced intimal and adventitial areas, after furin inhibition, are in accordance with the previous studies on TGF-β1 inhibition.[14,17-19] However, the previous observations on TGF-β1 inhibition showed less luminal narrowing and less arterial shrinkage. We found no changes in lumen areas, because the reduction in neointima was accompanied with a tendency to increased shrinkage of the artery (decreased vessel areas).

Although involvement of TGF-β in arterial remodeling has been reported, these results are confusing. Inward arterial remodeling can be inhibited using a TGF-β signalling inhibitor[14] while adenoviral overexpression of TGF-β (stimulating TGF-β signalling) also inhibits inward arterial remodelling.[20] This might be explained by the recent observation, that in endothelial cells TGF-β can activate distinct pathways. The Activin receptor-like kinase 5 (ALK5) - Smad 2/3 pathway stimulates collagen production but inhibits cell proliferation, migration and MMP production while the ALK1 - Smad1/5 pathway stimulates cell proliferation, migration and MMP production but inhibits collagen production.[21] This might also apply to the non-endothelial cells of the arteries. We found that both pathways are activated after balloon dilation. Since both pathways have different kinetics and threshold levels for TGF-β, and both pathways can influence each other, local levels will determine which pathway is dominant.

Active TGF-β levels were observed early after injury, whereas the increases in pSmad pathways last longer. This might be explained by the family of bone morphogenic proteins (BMP) or activin. Next to TGF-β, BMP can activate the Smad 1/5 pathway,[22] whereas activin can activate Smad 2/3.[23] The increased expression of activin was observed at 2, 4, 7 and 14 days after arterial injury,[24] whereas BMP can influence Smad phosphorylation of SMCs.[25] Moreover, activin overexpression

Angiographic	Empty-virus		alpha1-PDX		
	mean	sem	mean	sem	p
pre-BD	1494	100	1439	96	0.6
BD	2875	134	2761	58	0.26
post-BD	2324	71	2246	65	0.31
termination	1778	137	1666	79	0.54
LLL	-546	167	-580	84	0.87

Morphometric parameters	Empty-virus		alpha1-PDX		
	mean	sem	mean	sem	p
Outside adventitia	1886468	90858	1696488	103816	0.12
EEL	1362963	67273	1246639	89903	0.23
IEL	937285	70491	833116	94356	0.29
lumen	447554	61998	430134	73844	0.82

Table 2: *Angiographic diameter (μm) and morphometric area parameters (μm²) of the empty and α1-PDX virus treated arteries. The angiographic diameters are measured pre-dilation (pre-BD), during balloon dilation (BD) and post-dilation (post-BD), as well as at termination. Late lumen loss (LLL) is calculated by the diameter post-dilation minus the diameter at termination. The morphometric area parameters are measured at sections from each artery (N=9 rabbits). Angiographic diameters and morphometric areas did not differ significant between the two groups. (IEL=internal elastic laminae, EEL=externa; elastic laminae).*

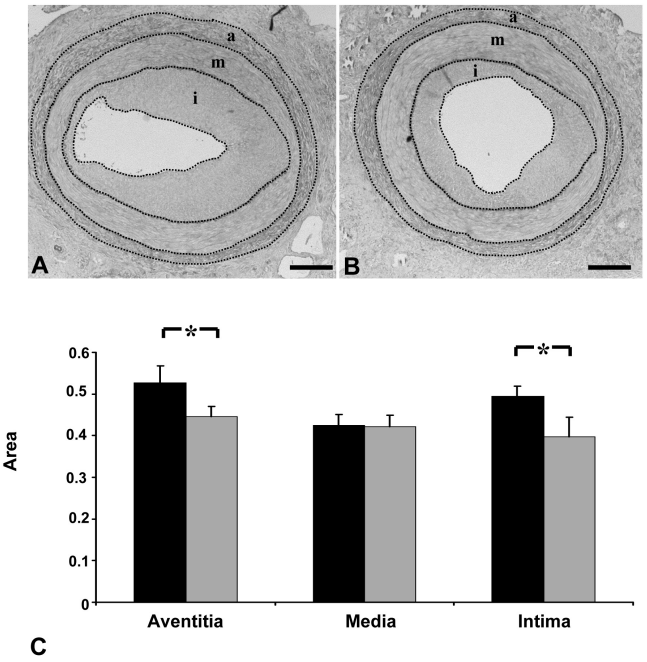


Figure 5: *Balloon dilated arteries were transfected with Ad.CMV.Empty (A) or Ad.CMV.PDX (B) and rabbits were terminated after 14 days. (a = adventitia, m= media, i = intima; Bar=250ìm) (C) Histomorphometric adventitial, medial, and intimal layer areas (mm²) in the empty (black) and α1-PDX (grey) treated arteries. (data are mean ± standard error of the mean ;N=9 rabbits, * = P<0.05). A significant reduction in adventitial and intimal area is observed after furin inhibition.*

inhibits neointima formation in mice and in cultured human saphenous vein segments.[26] Thus, based on our observation we cannot conclude which pathway will dominate in each layer predominantly.

In conclusion, our data show that furin, the MT1-MMP-MMP2 activation cascade and the TGF-β -Smad signaling pathway are activated after balloon injury. Advential furin inhibition shows that furin is involved in the arterial response to injury most likely via activation of the TGF-β -Smad signaling pathway and identifies furin as a possible target to inhibit intimal hyperplasia.

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6 Extracellular matrix metalloproteinase inducer (EMMPRIN) release is associated with MMP expression in arterial lesions

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ABSTRACT

Objective: *An extracellular matrix metalloproteinase inducer (EMMPRIN) was recently reported to stimulate the production of matrix metalloproteinases (MMPs) in oncological tissues. We studied whether EMMPRIN expression was modulated following arterial injury or in human atherosclerotic plaques, situations associated with an increased expression and activation of MMPs.*

Methods and Results: *Rabbit arteries were balloon dilated after which MMP-1, MMP-2, and MT1-MMP levels were increased after day 7 compared to contralateral control arteries. Similarly EMMPRIN mRNA levels were increased after 7 and 14 days. On the other hand, EMMPRIN protein levels were decreased following day 7. We confirmed the inverse relation between decreased EMMPRIN protein levels and increased MMP activity in human atherosclerotic plaques and demonstrated release of EMMPRIN in vitro. While EMMPRIN was present in human serum, no significant differences between serum levels of patients with clinically manifest atherosclerotic disease and healthy control patients were observed.*

Discussion: *Our results suggest that decreased EMMPRIN levels are associated with increased MMP tissue levels, potentially due to EMMPRIN release, in both experimentally injured arteries as well as atherosclerotic human tissue. On the other hand, our results suggest that serum EMMPRIN levels are not a useful marker of human atherosclerotic disease.*

SUBMITTED FOR PUBLICATION

INTRODUCTION

A molecule known as extracellular matrix metalloproteinase inducer (EMMPRIN), also called basigin, CD147, OX-47 or TCSF, has been recently identified as a tumor cell surface glycoprotein able to stimulate the production of different matrix metalloproteinases (MMPs), including MMP-1, MMP-2 and MMP-3.[1,2] Sequence analysis of EMMPRIN suggested two extracellular immunoglobulin domains, a transmembrane domain, and a cytoplasmic domain. EMMPRIN can be produced with different modes of glycosylation,[3] resulting in different molecular weights, but is in human tissue generally observed as a 58kDa protein. EMMPRIN glycosylation seems to be critical for MMP induction capability.[3,4] EMMPRIN expression was identified in human CD68+ macrophage-rich atheroma and was induced during in vitro monocyte-macrophage differentiation.[5]

The MMPs are a family of endogenous, zinc-dependent enzymes allowing for matrix remodeling and neointima formation in response to arterial injury[6,7] or for remodeling after sustained flow changes.[8] High levels of MMPs are also present in the vulnerable regions of human atherosclerotic plaques.[9] The potential connection between MMP expression and EMMPRIN in relation to atherosclerotic lesion formation and progression remains unknown.

We therefore investigated in parallel expression and localization of EMMPRIN and MMPs in arterial tissue in a rabbit model of lesion formation, as well as in human atherosclerotic plaques and serum from atherosclerotic patients.

MATERIALS AND METHODS

Animals

Animals were housed to conform to the Guide for the care and Use of Laboratory Animals (NIH publication No.85-23, 1985) and all experiments were approved by the ethical committee on animal experiments of the University Medical Center, Utrecht. Femoral and external iliac arteries of forty-two New Zealand White rabbits were unilaterally balloon dilated with a 3.0 mm balloon[10]. Geometrical remodeling and intimal formation were analyzed and arteries were harvested for immunohistochemical and biochemical analysis, as previously described.[10]

Immunohistochemistry

Frozen 5 μm sections of arterial segments were incubated with a pAb for EMMPRIN (1:100, Santa Cruz) or respectively a mAb for MMP-2 (1:50, clone 42-5D11, Calbiochem). Subsequently, sections were incubated with a rabbit-α-goat-biotin (DAKO) and a horse-α-mouse-biotin (Vector Laboratories), respectively, followed with streptavidin-peroxidase (Vector Laboratories) and developed using AEC in dimethylformamide, per manufacturer's protocol.

Quantitative RT-PCR

Rabbit EMMPRIN (Forward: 5'- tgcagctcacctgtaccttg -3'; Reverse: 5'- aggcaggag-tacttgccaaa -3') and ribosomal 18S (Forward: 5'-tcaacacgggaaacctcac-3'; Reverse:5'-acaaatcgctccagcaac-3') primers were designed using the Prime program at CMBI (Nijmegen).

Quantitative RT-PCR was performed as previously described.[10] The PCR reactions used, started with 2 min at 94°C followed by 40 cycles of: 30 sec at 94°C, 30 sec at 65°C (EMMPRIN) or at 50°C (18S) and 30 sec at 72°C. Data are presented as the ratio of the dilated and control arteries.

Western blotting and Zymography

For Western blotting, samples (8 µg) were loaded on 10% SDS-PAGE gels and transferred onto a Hybond-P membrane (Amersham). EMMPRIN was detected with a goat-α-human EMMPRIN (clone sc-9753, Santa Cruz) and a rabbit-α-goat-HRP (DAKO). MT1-MMP with a mouse-α-human MT1-MMP (clone113-5B7, Oncogene) and a goat-α-mouse-HRP (1:1000, DAKO), followed by chemiluminescence substrate (NEN™ Life Science Products) and exposed to the ChemiDoc XRS system (Biorad). Negative controls were run omitting the primary antibody or using an isotype control antibody.

SDS-PAGE Zymography was performed as described before.[11] For MMP-1, casein gels were used and quantified using the ChemiDoc XRS system (Biorad).

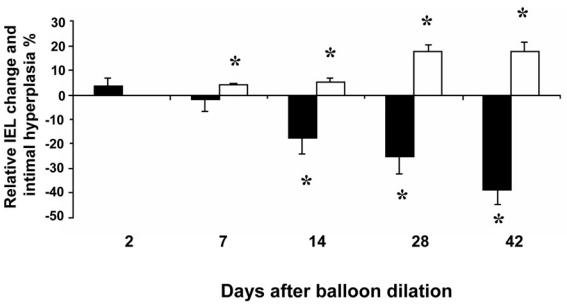


Figure 1: *Changes in the areas of internal elastic lamina (IEL, black bars) and neointimal lesion (white bars) at 2, 7, 14, 28 and 42 days after balloon dilation plotted as a percentage of the total IEL area relative to day zero (N = 6-8 rabbits per time-point, *= p<0.05).*

Metabolic labeling and EMMPRIN immunoprecipitation

Primary isolated adventitial fibroblasts (PAF; rabbit) were cultured in MEM (10% FBS) in 6 wells plate. Cells were starved, labeled (16 hour) in D-MEM (Gibco Invitrogen Corporation), 1% FBS, and Pro-mix L-[^{35}S] in vitro cell labeling mix (Amersham Biosciences). Culture medium and cell lysate (obtained by 30 min extraction of monolayers in ice-cold 50mM Tris-HCL, pH8.0, 150mM NaCl, 1% NP-40) were collected at 4 °C for immunoprecipitation of EMMPRIN, using 2µl

antibody (sc-9753, Santa Cruz)/sample and 10% protein A sepharose CL-4B (Amersham Biosciences). Medium was centrifuged at 15.000 rpm for 15 min to lose any potential cell debris. After washing, part (20%) of the precipitates was separated on 10% SDS-PAGE, and radioactivity was detected by film (Kodak).

Human atherosclerotic tissues

Carotid artery plaques were obtained from Athero-express, an ongoing multi center study in which carotid atherosclerotic specimen are obtained from patients undergoing endarterectomy (see http://www.vascularbiology.org/athero-express.htm). Atherosclerotic plaques from patients (n=65, 42 male and 23 female, mean age 69.1 ± 8.1) were collected on ice and protein was isolated as described above. Subsequently, we performed zymography and analyzed total MMP-2 and MMP-9 activity. We selected samples from the highest (3.98-5.85 arbitrary units) and lowest quartile (0.85-1.26 arbitrary units) of MMP activity (10 patients each). The selected samples were used for EMMPRIN Western blotting as described above.

Quantification of EMMPRIN by ELISA

Human EMMPRIN was measured in serum samples by ELISA as described before.[12] We determined EMMPRIN levels in serum of patients with severe atherosclerotic disease (n=80, 40 males and 40 females, mean age 59.7) and a control group. All patients originated from the Second manifestation of ARTerial disease

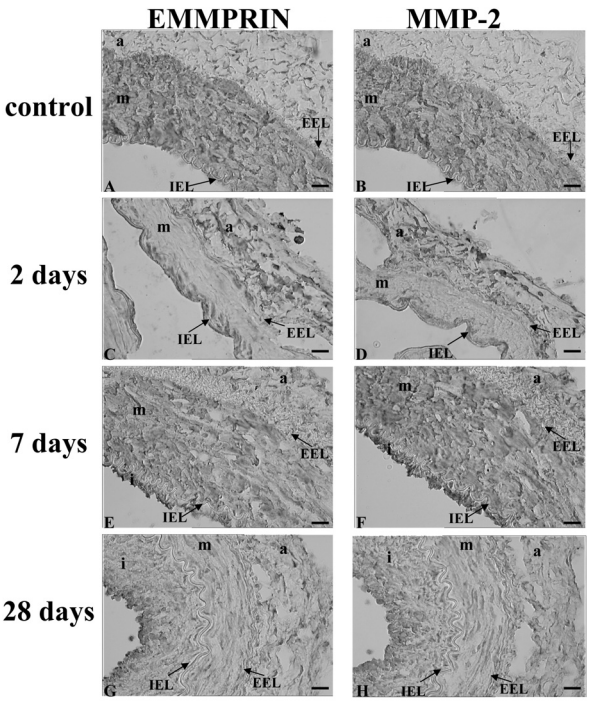


Figure 2: *Localization of EMMPRIN and MMP-2 in cross sections of normal (contralateral control) and balloon injured rabbit arteries at 2, 7 and 28 days after balloon dilation. (bar= 500 μm, a= adventitia, m= media, i= intima, IEL= internal elastic lamina, EEL= external elastic lamina). Note the similar localization of EMMPRIN and MMP-2 at all times investigated.*

(SMART) study.[13] Selection criteria and characteristics of the studied population have been described before.[14] Control patients were matched for risk factors (hypertension, diabetes, hypercholesterolemia, age, gender) and did not suffer from clinically manifest atherosclerotic disease (n=77, 38 males and 39 females, mean age 57.5).
Briefly, serum samples pretreated with 10% protein G sepharose (SIGMA;P-3296) were added to microtiter plates coated with goat-α-human EMMPRIN (R&D systems). After removal of unbound protein by washing, biotinylated goat-α-human EMMPRIN (400ng/ml, R&D systems) was added, followed by streptavidin horseradish peroxidase (R&D systems) and, ortho-phenyl diamine substrate (PIERCE). The optical absorbance was measured at 490nm.

Statistical analysis
Statistical analysis of the date obtained from the experimental model was performed using a Wilcoxon matched pairs signed rank sum test and data are presented as ratio operated versus control mean ± the standard error of the mean. The Mann-Whitney U test was performed to analyze the potential differences in expression in human atherosclerotic tissues and a t-test for differences in EMMPRIN levels in the serum (data are presented as mean ± the standard error of the mean). P values of < 0.05 were considered as statistical significant.

RESULTS

Experimental arterial remodeling in response to balloon injury
An initial tendency towards the increase in average arterial internal elastic lamina (IEL) area was detected (+3.5%) at two days after balloon dilation, however, from day 7 we measured a decrease in IEL (corrected for neointima formation), which became statistically significant at day 14 (-17.7%, p=0.05) (Figure 1). At day 28, the IEL had decreased by -25.5% (p=0.01) and at day 42 by –38.6% (p=0.0005). The onset of neointima formation was also detectable at day 7 (1.5% increase in intimal area) and this increased till day 28 (16.7%), when it reached a plateau till day 42 (Figure 1). The neointimal lesion contained mainly smooth muscle cells (SMC) with only a few macrophages detected in the artery at all time points (not shown). As expected no neointima was detected in contralateral uninjured arteries.

EMMPRIN and MMP-2 expression and localization in situ
In control (uninjured) arteries, EMMPRIN as well as MMP-2 expression was mainly localized in adventitial and endothelial cells and a diffuse staining was present in the medial layer (Figure 2A and B). A similar pattern was still present two days after balloon dilation, however there was a lack of staining in the medial layer, probably due to necrosis of medial smooth muscle cells (Figure 2C). After 7 days, a diffuse EMMPRIN staining was detected in the developing neointima (figure 2E), and after

28 days most of the staining was present at the luminal side of the neointima (figure 2G) and did not change at 42 days (data not shown). At all time points, the positive signal for MMP-2 was localized in the same regions as that of EMMPRIN (Figure 2D-F-H). No staining was detected in the controls for immunocytochemistry (no primary antibodies, data not shown).
Quantitative RT-PCR, used to measure EMMPRIN mRNA tissue levels, indicated

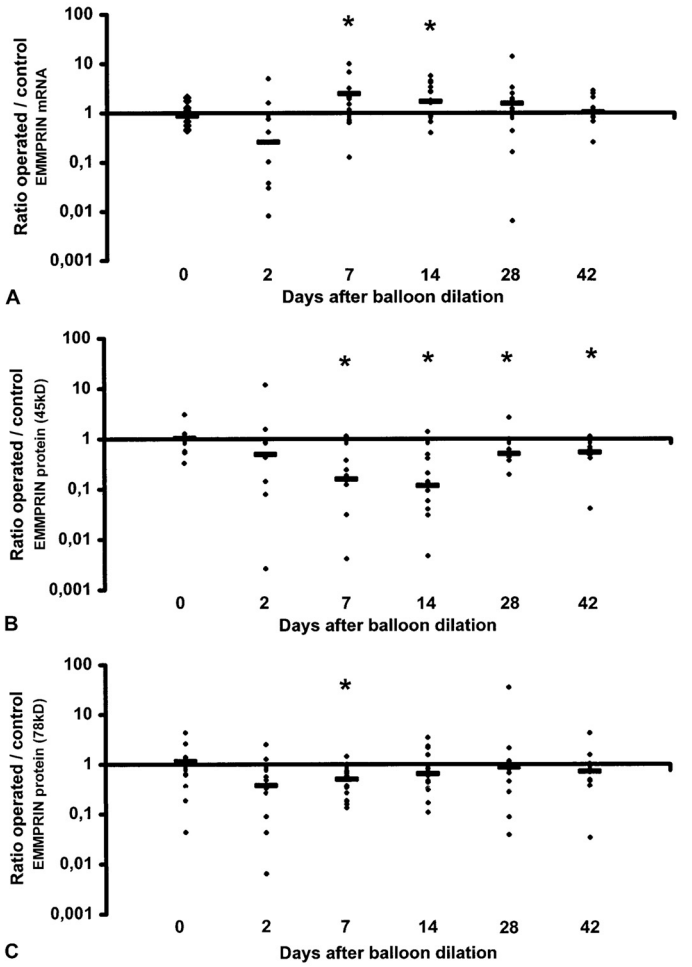


Figure 3: *EMMPRIN expression patterns 2, 7, 14, 28 and 42 days after balloon dilation. Relative mRNA (A) and protein (B: 45kD and C: 78kD) expression of balloon dilated arteries. Data are presented as the ratio between levels in operated / control for each rabbit (dots) including the median at each time point (line). (N=6-8 rabbits per time point, * = P<0.05). Statistically significant differences were detected in specimens collected 7 days after balloon injury. Interestingly, while EMMPRIN mRNA levels were increased, protein levels were decreased in the same samples.*

that in injured arteries the levels were increased at day 7 (p=0.05) and 14 (p=0.04) after the balloon dilation, and returned to basal levels after 42 days (Figure 3A), compared to the contralateral (uninjured).
The levels of the 45kD form of EMMPRIN, determined by Western blotting (Figure 3B), were significantly decreased at day 7, 14, 28, and 42 (p=0.007, p=0.009, p=0.02, and p=0.02, respectively). The levels of the 78kD EMMPRIN form were decreased at day 7 (p=0.002), and returned to basal levels after 28 days (Figure 3C).

MMP-1, MMP–2 and MT1-MMP levels after balloon dilation
We used zymography and Western blotting to study MMP activation and expression in the specimens of balloon-dilated or contralateral control arteries (Figure 4). MMP-1 levels were significantly increased in the balloon dilated arteries after 7 days (p=0.01), reached a maximum at 14 days (p=0.003) and remained high until day 42 (p=0.02) (Figure 4A). Injury was associated with early significant activation of

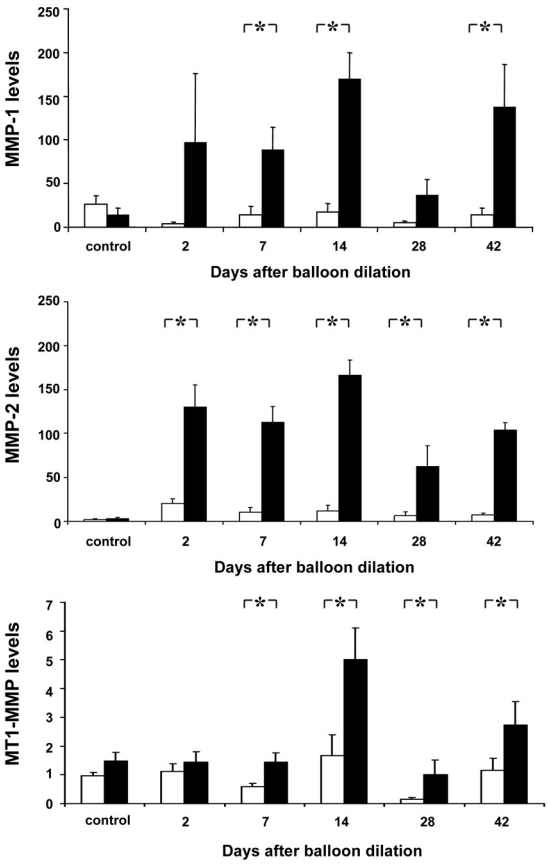


Figure 4: *Injury increases levels of latent and activated MMP-1, MMP-2 and MT1-MMP. Expression of MMP-1 (A), activated MMP-2 (B), and activated MT1-MMP (C) was calculated relative to levels detected from control contralateral arteries (White bars = control arteries, black bars = balloon-dilated arteries; data are presented as the measured mean number of pixels ± sem) (N=6-8 rabbits per time point, * = p<0.05).*

MMP-2 at two days (p=0.004), which continued to increase, reaching maximum at 14 days (p=0.002), then leveling off for the remainder of the study period (Figure 4B). Levels of activated MT1-MMP were significantly increased at day 7 (p=0.003), reached a maximum at 14 days (p=0.038), and also remained elevated until 42 days post balloon injury (p=0.016) (Figure 4C).

EMMPRIN expression in vitro

To address the potential release of EMMPRIN protein we performed in vitro metabolic labeling of cultured primary rabbit adventitial fibroblasts and subsequently investigated cell-associated and soluble forms by immunoprecipitation and separation on 10% SDS-PAGE. We found both forms of EMMPRIN (45kD and 58kD) to be present in the total cell lysate, as in the culture medium of cells, showing that EMMPRIN was released by the cells (Figure 5).

EMMPRIN expression in human atherosclerotic plaques

We selected tissue samples of human atherosclerotic tissue that had high and low levels of MMP activity (10 patients each) and then examined these for EMMPRIN levels by Western blotting (Figure 6A). We detected EMMPRIN positive signal corresponding to 45kD, 58kD, and 78kD forms. The levels of the 45kD form were decreased in the group with high MMP levels (p=0.03, figure 6B), while the other (58kD and 78kD) forms were not significantly different among the two groups (p=0.73 and p=0.54, respectively, online figure I)

EMMPRIN levels in human serum

Human EMMPRIN (45kD and 58kD) was detected by Western blotting using the antibody used in the ELISA (online figure Ia, lanes 2+4). On the other hand, this antibody did not cross-react with rabbit EMMPRIN (online figure IIa, lanes 3, 5 + 6). A linear response was obtained in the ELISA with the recombinant human EMMPRIN standards (online figure IIb). There were no differences (p= 0.4) in EMMPRIN

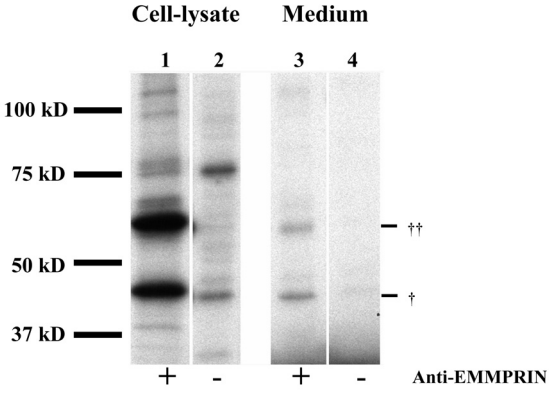


Figure 5: *Immunoprecipitation of EMMPRIN from primary isolated adventitial fibroblasts after metabolic labeling. The same two forms of EMMPRIN were present in the cell lysates (lane 1) as well in the culture medium (lane 3). Control samples processed in the absence of anti-EMMPRIN antibody (-) do not show the typical bands (lane 2 and 4). (†= 45kD, ††=58kD).*

serum levels of patients with severe atherosclerotic diseases (114.3 ± 13.2) compared to the levels in a control group without clinically manifest disease (131.7 ± 17.8) (Figure 6C).

DISCUSSION

The potential role in the arterial response to injury and development of atherosclerosis of EMMPRIN, an extracellular matrix metalloproteinase inducer recently reported to be localized in the human atheroma, is still unknown.

We studied the effect of balloon dilation injury, known to trigger formation of arterial lesion, upon the expression and localization of EMMPRIN. In parallel, effects upon MMP-1, MMP-2, and MT1-MMP were assessed. We confirmed that intimal hyperplasia was present from day 7 and inward arterial remodeling occurred from day 14 after balloon dilation.[10] We found that injury was followed by early increases in the levels of MMP-1 and activated MMP-2 (day 2), followed by increased levels of activated MT1-MMP at day 7. In the same specimens, we found that EMMPRIN mRNA levels were significantly increased at 7 and 14 days after balloon dilation. In terms of arterial wall distribution, immunohistochemistry indicated that EMMPRIN co-localized with MMP-2 in control arteries and at all time points after the injury. Co-localization of EMMPRIN and MMP-9 was reported by a previous study of human atherosclerotic lesions, [5] which suggested a possible role for EMMPRIN in plaque growth and destabilization. In contrast, the lesions that develop in response to balloon injury in our rabbit model contain hardly any macrophages, being mostly driven by intimal smooth muscle cell hyperplasia and adventitial fibroblast contraction. Our observations suggest that vascular cells can also upregulate expression of EMMPRIN during the remodeling response to injury and lesion formation.

Interestingly, EMMPRIN protein levels were affected in the opposite direction with EMMPRIN mRNA levels in response to arterial injury, i.e. we found decreased protein levels from day 7. One possible explanation for this discrepancy between EMMPRIN mRNA and protein levels was that while more protein was produced, tissue levels were decreased through “loss” of cell- associated protein. This potential process would be in concordance with the previously proposed hypothesis that EMMPRIN induces MMP expression either by a cell-cell interaction or through a paracrine-mediated effect.[4,5] In in vitro experiments with rabbit primary adventitial fibroblasts, we detected newly synthesized EMMPRIN both associated with cells and released in the culture medium. The presence of full length EMMPRIN in conditioned medium of breast cancer cells was also described by Tayler et al.[15] They also reported that EMMPRIN immuno-depleted culture medium was unable to activate MMP-2 release in fibroblasts. We suggest that EMMPRIN is released after arterial injury and stimulate adjacent cells to produce MMPs. Escape of soluble EMMPRIN from arterial lesions into the circulation would result in decreased arterial tissue

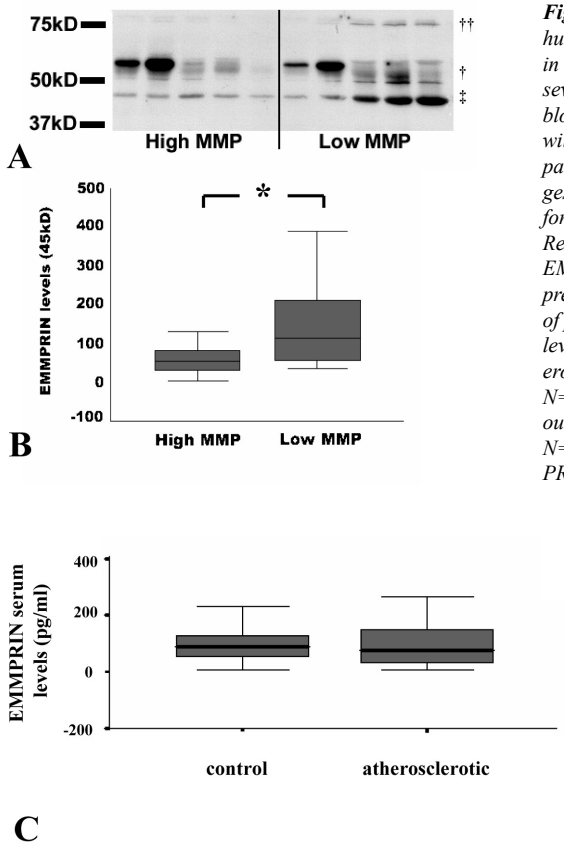


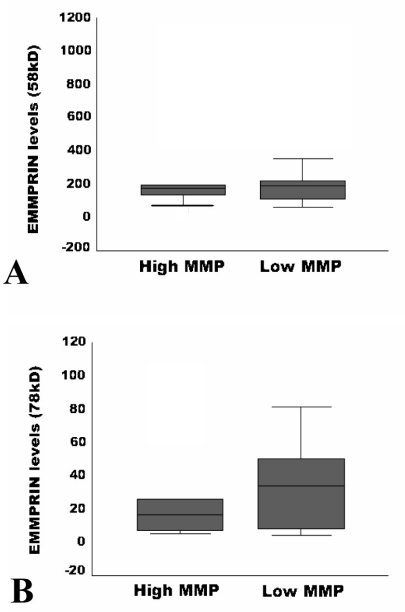
Figure 6: *EMMPRIN expression levels in human atherosclerotic tissue samples and in serum from patients with and without severe atherosclerotic disease. A: Western blot analysis of EMMPRIN for patients with high and low levels of MMPs (5 patients illustrated for each group) suggested a different distribution of the three forms (‡=45kD, ‡ ‡=58kD, ††=78kD). B: Relative expression levels of 45kD form of EMMPRIN (10 patients for each)(data are presented as the measured mean number of pixels ± sd, * = p<0.05). C: EMMPRIN levels in serum of patients with severe atherosclerotic disease ("atherosclerotic", N=80) compared to a control group without clinically manifest disease ("control", N=77) (data are presented as mean EMMPRIN levels (pg/ml) ± sd).*

levels of EMMPRIN. The association between decreasing EMMPRIN protein levels and increased MMP activity, found in the rabbit model of lesion formation, was also confirmed in human atherosclerotic plaques. However, serum levels of EMMPRIN did not differ between control and atherosclerotic patients, thus our results do not support the utility of serum EMMPRIN as an indicator of MMP activity in the lesions or a marker of atherosclerotic disease. It is worth noting that while plasma MMP-9 concentrations were previously reported as a marker for future cardiovascular mortality[16] and increased MMP-2 and MMP-9 serum levels were detected in patients with acute coronary syndromes[17], a correlation between serum levels and arterial tissue levels of MMP activity in the same patient remains to be demonstrated.

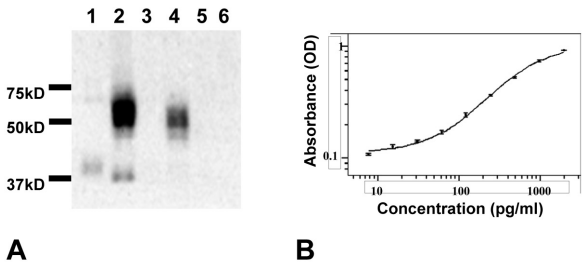
Several potential reasons for the apparent discrepancy between EMMPRIN and MMP protein expression in the tissue and serum could be formulated. First, it is possible that increased mRNA levels do not get translated into increased protein production. Or, if more EMMPRIN protein is indeed produced but released, it may be uptaken by the neighboring cells or degraded through interaction with MMPs or exhausted by some other mechanism, both of which would be consistent with low-

ered tissue protein levels and no effect upon serum levels. Other potential reasons for which we did not detect differences in EMMPRIN serum levels might be related to the difficulty to perform such clinical studies. While we perform the analysis of the lesions harvested from the patients by endarterectomy, we do not have additional information regarding all other plaques that patients may have, i.e. stable vs. unstable, which would require further in vivo imaging, not a routine diagnostic performance. Second, since the progression of atherosclerotic disease is a continuous ongoing process, not always accompanied by clinical manifestations, we might miss the stage at which EMMPRIN levels were elevated. Furthermore, selection of the control group based on the levels of their known risk factors instead of having a healthy group might also influence the outcome of the results. This would be similar to the case in which Beaudeux et al.[18] reported that elevated MMP-3, MMP-9 and TIMP-1 serum levels in patients with atherogenic hyperlipidemia compared to normolipidemic control subjects did not differ between patients with and without atherosclerotic lesions. However, in our study all the samples were obtained from the SMART study[13], where all the participants undergo screening for clinically silent peripheral atherosclerotic disease. None of the control patients suffered from atherosclerotic disease in the carotid artery (duplex measurement), aneurysm formation (echo) or decreased ankle arm indices. Hence, it is not likely that clinically silent atherosclerotic disease in the control group will explain our observations. Third, conventional medical treatment was shown to decrease MMP-2 and MMP-9 levels over time in patients with unstable angina.[17] Also, EMMPRIN release is calcium dependent, thus may be influenced by the treatment with calcium inhibitors.[19]

We detected two different forms of EMMPRIN which size is species dependent:



Online figure I: *Relative expression levels of 58kD (B) and 78kD (C) forms of EMMPRIN in human atherosclerotic tissue samples with relative high and low levels of MMPs (10 patients for each)(data are presented as the measured mean number of pixels ± sd,).*



Online figure II: *EMMPRIN expression levels detected by ELISA. A: Western blot of EMMPRIN with the antibody used in the ELISA. Lane 1= recombinant human EMMPRIN (5ng) used to establish the standard curve, lane 2= lysate human THP-1 cells (8 μg), lane 3= lysate rabbit PAF (8 μg), lane 4= lysate human vascular SMC (8 μg), lane 5+6= lysates balloon dilated rabbit arteries (8 μg). B: Standard curve of the ELISA for EMMPRIN. A linear response was obtained with the recombinant EMMPRIN (OD measured at 490 nm).*

45kD and 78kD for rabbit; 45kD and 58kD for human tissue samples. Different forms of EMMPRIN based on degree and sites of glycosylation were described before[3]. Similarly EMMPRIN forms may vary during development, which may explain the differences we detected between EMMPRIN from rabbit arterial tissue (45kD and 78kD) or rabbit fibroblast cell culture (45kD and 58kD). In addition, differences may be related to the fact that the rabbit fibroblasts were studied under basal conditions.

Variation in EMMPRIN mRNA levels in the lesions developing in response to injury are consistent with the time course we reported previously in the rabbit model of lesion formation[10], where the increase of Moesin, a protein associated with cell migration, occurred after the first week after balloon dilation, with a maximum at 14 days. MMP activity required for cell migration is also maximal at day 14. The time and spatial association of EMMPRIN expression within the arterial wall suggests that EMMPRIN may play a role in cell migration after injury. Yang et al. reported that increased EMMPRIN expression has a role in the migration of cancer cells in an in vitro invasion assay.[20] However, a causal relationship between EMMPRIN expression, MMP induction, and atherosclerotic lesion formation and progression warrant further investigations using specific inhibition of EMMPRIN such as it could be obtained using a genetically deficient animal model.

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7 Association of MMP-2 levels with stable and MMP-9 levels with unstable lesions in human endarterectomies. A role for different EMMPRIN glycosylation forms

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ABSTRACT

Introduction: *Matrix metalloproteinase (MMP) synthesis and activation are important features in atherosclerotic plaque destabilization. Extracellular matrix metalloproteinase inducer (EMMPRIN) stimulates the expression of MMPs, whereas furin is involved in the activation of proproteins, including some MMPs. We studied MMP-2 and -9, EMMPRIN and furin levels in relation to plaque characteristics in human atherosclerotic plaques.*

Methods: *The culprit lesions of atherosclerotic plaques (N=150) were examined and categorized as fibrous (0-10% fat), fibrous-atheromatous (10-40% fat), or atheromatous (above 40% fat). In addition, collagen, smooth muscle cell (SMC), and macrophage stained sections were analyzed semi-quantitatively and categorized as no/minor or moderate/heavy. Adjacent segments were used to isolate total protein to assess MMP-2 and MMP-9 levels (zymography) and EMMPRIN and furin levels (Western blotting).*

Results: *Macrophage rich lesions revealed higher MMP-9 levels ($p=0.01$), whereas SMC rich lesions showed higher MMP-2 levels ($p=0.01$). The expression of more glycosylated EMMPRIN (58kD) and furin were not related to plaque characteristics. The levels of less glycosylated EMMPRIN (45kD) was higher in SMC rich lesions ($p=0.005$), and lower in macrophage rich plaques ($p=0.03$). Interestingly, regression analysis revealed that EMMPRIN 45kD was related to pro-furin and MMP-2 levels (all $p<0.005$), whereas EMMPRIN (58kD) was related to active furin and MMP-9 levels (all $p<0.001$).*

Conclusions: *MMP-9 levels were associated with more inflammatory lesions, whereas MMP-2 levels were higher in stable plaques. Different EMMPRIN forms were associated to other MMP and furin levels. Because differences in EMMPRIN forms were also observed among plaque phenotypes, EMMPRIN may play a role in MMP regulation and the development of atherosclerosis and plaque destabilization.*

SUBMITTED FOR PUBLICATION

INTRODUCTION

Atherosclerosis is an inflammatory disease characterized by remodeling of the extracellular matrix (ECM) and the accumulation of lipids and inflammatory cells in the arterial wall.[1,2] Advanced stable atherosclerotic plaques are rich in collagen and SMCs, while a thin fibrous cap with a dense inflammatory infiltrate and a large lipid core characterize unstable and ruptured plaques.[3] Increased expression of Matrix Metalloproteinases (MMPs)-1, -2, -3, -7, -8, -9, and –13 are found in macrophages bordering the lipid core adjacent to the fibrous cap and in SMCs covering the shoulder regions of atherosclerotic plaques, which are sites that seem more prone to rupture.[4-7] MMPs are involved in the breakdown of the matrix and facilitate cell migration or the activation of other proteins.[8,9] It is well appreciated that MMPs contribute to ECM destruction in cardiovascular pathologic processes like de novo atherosclerotic plaque progression.[10] MMPs are implicated to contribute to plaque vulnerability, by degradation of the fibrous cap. However, regulation of MMP expression and subsequent activation and how these are related to plaque instability is not clear.

A recently identified protein, called extracellular matrix metalloproteinase inducer (EMMPRIN), stimulates the production of different matrix MMPs, including MMP-1, MMP-2 and MMP-3.[11-13] Previously we found that EMMPRIN expression is induced after arterial injury.[14] In another study, EMMPRIN was identified in human CD68+ macrophage-rich atheroma, and during in vitro monocyte-macrophage differentiation.[15] However, the relation of EMMPRIN with plaque characteristics is not investigated in a larger population and also its association with MMP expression and activity in the atherosclerotic plaque has not been described.

Furin is a member of the proprotein convertases and a proteolytic activator of proproteins.[16] Furin is described to be involved in the arterial response to injury.[17] Several candidate substrates have been identified, including growth factors, cell surface receptors, coagulation factors, extracellular matrix proteins, and MMPs.[18,19] The relation of furin with atherosclerotic plaque composition has not been studied previously.

Both EMMPRIN and furin could be potential therapeutic targets to inhibit downstream matrix breakdown and subsequent plaque stabilization. However, although their function has been examined in vitro, expression studies in atherosclerotic plaques are lacking and their relation with a plaque phenotype unknown.

In the present study, we examined human atherosclerotic plaques that were obtained by endarterectomy. Plaque characteristics were associated with expression levels of MMP-2 and MMP-9, EMMPRIN, and furin. Furthermore, EMMPRIN expression levels were associated with furin and MMP levels.

MATERIALS AND METHODS

Atherosclerotic specimen

Athero-Express is an ongoing multi center study in which carotid atherosclerotic

specimen are obtained from patients undergoing endarterectomy in the University Medical Centre Utrecht and the Sint Antonius hospital Nieuwegein. Objective of this prospective follow-up study is to assess tissue bio-markers that are associated with future development of restenosis and adverse cardiovascular events. The ethics committees of the participating hospitals have approved the study and all subjects gave a written informed consent. (see also http://www.vascularbiology.org/atheroexpress.htm)
The excised plaques were rinsed in saline and divided in segments of 0.5 cm. The culprit lesion containing the largest plaque is defined as segment 0 and used for histological plaque characterization. The adjacent segment (+1) is used to determine expression levels.

Histological analysis
For histological analysis, segment 0 was formalin fixed, paraffin embedded and slices of 5 um thickness were obtained and stained routinely. All stained sections were analyzed by two observers. A third independent observer also analyzed all sections if interpretations differed between observer 1 and 2. The following stainings were performed to characterize the plaque: Picro Sirius red (collagen using normal light/polarized light), CD68 (macrophages), alfa actin (smooth muscle cells), haematoxilin and Elastin von Giesson. The CD68 and alfa actin stains are performed fully automated (Ventura, Tucson, USA).
Each plaque was studied semi-quantitatively for collagen content (along luminal border), smooth muscle cells (SMCs), macrophages (clusters of more than 10 cells) and calcifications. Both observers scored all sections as no/minor or moderately/heavily stained. Moreover, for each plaque an overall plaque phenotype was assigned (fibrous – fibroatheromatous – atheromatous).

Protein expression
The adjacent segment (+1) was used to isolate total protein by using Tripure™ Isolation Reagent (Boehringer Mannheim) according the manufacturers protocol. Isolated protein concentrations were determined using the Biorad DC Protein Assay (Microplate Assay Protocol).
To determine pro and active MMP-2 and MMP-9 levels in each plaque, zymography was performed as described before.[20] In short, protein samples (1 ìg) were separated on a 10% SDS–PAGE gel containing 1 mg/ml gelatin (Sigma). After incubation overnight at 37°C in Brij solution (0.05 M Tris–HCl pH 7.4, 0.01 M CaCl2, 0.05% Brij 35 (Sigma)), the gel was stained (25% methanol, 15% acetic acid, 0.1% Coomassie blue) and proMMP-2, active MMP-2, pro-MMP-9, and active MMP-9 bands were analyzed using the ChemiDoc XRS system (Biorad). Bands were characterized by size and co-migration of recombinant MMP-2 and MMP-9 protein. MMP expressions were normalized to recombinant MMP forms.
EMMPRIN expression levels were determined by Western blotting.[14] In short, 8 µg total protein was separated on a 10% SDS-PAGE gel and transferred onto a Hybond-P membrane (Amersham). The membrane was incubated with a polyclonal antibody

for EMMPRIN (1:500, clone sc-9753, Santa Cruz) and a rabbit-α-goat-horse radish peroxidase (1:1500, DAKO).
For furin expression, 10 μg total protein was separated on an 8% SDS-PAGE gel and transferred to an ECL membrane (Amersham). The membrane was incubated with a monoclonal antibody for furin (1:500, MON-148 Alexis Biochemicals), a biotinylated horse-α-mouse (1:1000, Vector Laboratories), and a streptavidin-horse radish peroxidase.
Chemiluminescence substrate (Sigma) and the ChemiDoc XRS system (Biorad) were used to detect and analyse EMMPRIN and furin expression. Control incubations did not reveal a signal. Also EMMPRIN and furin expression were normalized to a standardized sample that co-migrated on the different blots.

	CD 68			Alpha actin			Collagen		
	-	+	p	-	+	p	-	+	p
MMP-2 act	0.51 +/- 0.39	0.49 +/- 0.39	0.66	0.55 +/-0.39	0.47 +/- 0.38	0.20	0.45 +/- 0.40	0.52 +/- 0.39	0.24
proMMP-2	3.47 +/- 1.73	3.40 +/- 1.62	0.95	2.95 +/- 1.54	3.71 +/- 1.66	0.01*	3.01 +/- 1.98	3.54 +/- 1.57	0.17
MMP-9 act	0.30 +/- 0.38	0.57 +/- 0.64	0.01*	0.60 +/- 0.65	0.36 +/- 0.46	0.002*	0.61 +/- 0.65	0.38 +/- 0.49	0.02*
proMMP-9	0.58 +/- 0.66	0.92 +/- 0.90	0.01*	0.91 +/- 0.90	0.68 +/- 0.75	0.08	0.99 +/- 0.88	0.68 +/- 0.77	0.03*

Table 1: *MMP expression levels in sections stained for CD68 (macrophages), alpha actin (smooth muscle cells), and collagen. (All values are in mean +/- SD. *p<0.05. - = no/minor staining, + = moderate/heavy staining).*

Immunohistochemistry
Paraffin sections of atherosclerotic lesions were deparaffinized and boiled in citrate buffer (10mM, pH 6.0) to make the EMMPRIN epitope accessible for the antibody. Next, sections were incubated with the polyclonal antibody for EMMPRIN (1:100, clone sc-9753, Santa Cruz) and, subsequently, sections were incubated with biotinylated rabbit-α-goat (1:1000, DAKO) and streptavidin-horse radish peroxidase, followed by treatment with diaminobenzidine/nickel substrate. Control incubations did not reveal a signal.

Cell culture
A human cell-line of smooth muscle cells (SMCs, CRL-1999, ATCC) and monocytes (THP-1, TIB-202, ATCC) were cultured according the manufacturer. Cells were cultured under basal conditions and after washing in PBS, total cell protein was obtained by using Tripure™ Isolation Reagent (Boehringer Mannheim) as described above. Equal amount of cells were used for isolation and 5 μg total protein was separated to study EMMPRIN expression as described above.

Statistical analysis
Data are presented as mean +/- standard deviation. Oneway ANOVA was used to compare differences between fibrous, fibro-atheromatous, and atheromatous plaques. A Mann-Whitney Test was used to compare differences in expression between minor and heavily stained sections. Pearson uni-variate and multi-variate regression analyses were performed to compare EMMPRIN with furin and MMP levels. P values of < 0.05 were considered as statistically significant.

RESULTS

A total of 150 atherosclerotic lesions were histologically and biochemically analyzed. Plaque characteristics were compared to pro and active MMP-2 and MMP-9, two forms of EMMPRIN (45kD and 58kD), that differ in degree of glycosylation of the protein core (27kD), and to pro and active furin levels.

Plaque characteristics

Plaque characteristics are summarized in tables 1, 2, and 3. Plaques that revealed CD68 staining showed increased pro and active MMP-9 levels (both p=0.01). On the other hand, lower active MMP-9 levels (p=0.002), and a tendency towards low values for proMMP-9 (p=0.08) were observed when alpha actin staining was evident. Also, lower values for active and proMMP-9 were observed in sections with minor collagen staining (p=0.02 and p=0.03, respectively). Furthermore, proMMP-9 levels were increased (p=0.03) and active MMP-9 revealed a trend to increase (p=0.07) in more atheromatous plaque phenotype.
The observed proMMP-2 levels were lower when a more atheromatous phenotype was addressed to the lesions (p=0.01) and proMMP-2 expression levels were more evident when more SMC staining was observed (p=0.01).
No differences in expression of EMMPRIN 58kD and active and pro furin levels were observed between groups. However, lower EMMPRIN 45kD expression levels were found in heavily stained macrophage sections (p=0.03), moreover higher EMMPRIN 45kD expression was observed in heavily stained SMC sections (p=0.005). We did not observe any differences in EMMPRIN, furin and MMP expression levels between the more or less calcification containing lesions.

	CD 68			Alpha actin			Collagen		
	-	+	p	-	+	p	-	+	p
EMMPRIN 45kD	30.1 +/- 40.8	19.4 +/-27.1	0.03*	20.8 +/- 31.5	26.8 +/- 36.4	0.005*	20.5 +/- 28.7	25.8 +/- 36.4	0.23
EMMPRIN 58kD	65.0 +/- 92.7	78.3 +/- 119.6	0.79	89.3 +/- 114.8	61.7 +/- 101.8	0.39	85.6 +/- 110.3	67.7 +/- 106.4	0.32
Furin inact	116.0 +/- 98.4	113.8 +/- 79.0	0.61	222.5 +/- 117.9	225.4 +/- 125.1	0.97	208.9 +/- 121.9	228.5 +/- 122.3	0.37
Furin act	230.6 +/- 133.6	219.8 +/- 110.1	0.88	110.8 +/- 81.4	117.6 +/- 92.9	0.66	108.1 +/- 62.7	116.9 +/- 94.6	0.94

Table 2: *EMMPRIN and furin expression levels in sections stained for CD68 (macrophages), alpha actin (smooth muscle cells), and collagen. (All values are in mean +/- SD. *p<0.05. - = no/minor staining, + = moderate/heavy staining).*

EMMPRIN expression levels

Univariate analysis revealed a significant correlation between EMMPRIN 45kD and active and proMMP-2 levels (p=0.027 and p=0.045, respectively) (Table 4). Moreover, also a relation with pro-furin levels was observed (p<0.001). In a multivariate model, EMMPRIN 45kD was still associated with levels of pro- and active MMP-2 forms (p<0.001 and p=0.004) and pro-furin levels (p=0.003) within atherosclerotic plaque (Table 4).
Using univariate analysis, EMMPRIN 58kD was associated with proMMP-2

	Fibrous	Fibro-atheromatous	Atheromatous	p
MMP-2 act	0.46 +/-0.33	0.58 +/- 0.46	0.46 +/-0.36	0.22
proMMP-2	3.65 +/- 1.69	3.76 +/- 1.74	2.84 +/- 1.43	0.01*
MMP-9 act	0.31 +/- 0.40	0.49 +/- 0.61	0.55 +/- 0.60	0.07
proMMP-9	0.53 +/- 0.62	0.94 +/- 1.01	0.84 +/- 0.73	0.03*
EMMPRIN 45kD	26.5 +/- 39.9	25.8 +/- 29.5	20.9 +/- 33.8	0.68
EMMPRIN 58kD	54.3 +/- 94.6	71.6 +/- 115.5	91.4 +/- 109.3	0.22
Furin inact	107.5 +/- 100.5	131.6 +/- 88.9	107.4 +/- 72.8	0.33
Furin act	211.3 +/- 126.0	258.4 +/- 130.7	207.8 +/- 104.0	0.09

Table 3: *Semi-quantitative expression levels in fibrous, fibroatheromatous, and atheromatous sections (All values are in mean +/- SD. *p<0.05).*

(p=0.001), active MMP-9 (p=0.022), and the active and pro-furin levels (p=0.003 and p=0.045, respectively) (Table 5). Multivariate analysis revealed a strong relation of EMMPRIN 58kD with active and proMMP-9 (p<0.001 and p=0.001, respectively), and with active furin levels (p=0.001). Also a relation is found with proMMP-2 (p=0.026)(Table 5).

EMMPRIN expression in situ

The EMMPRIN protein analyses suggested that the 45kD band might originate from SMC rich plaques while the 58kD band was more prevalent in plaques that hide macrophages. Figure 1 shows representative sections stained for CD68 (macrophages, figure 1A+C) and alpha actin (smooth muscle cells, figure 1B+D). Immunoreactivity to EMMPRIN in the atherosclerotic lesions was observed in the macrophage-rich areas (figure 1E). However, immunoreactivity was also observed in smooth muscle cells (figure 1F), suggesting that both cell types can produce EMMPRIN. This was confirmed by western blot analysis on cell-lysate of cultured SMCs and macrophages (figure 2). Interestingly, the cell-lysate of macrophages contained relatively more EMMPRIN 58kD than the SMCs. Both cell types produce less EMMPRIN 45kD compared to EMMPRIN 58kD, however SMCs produced relatively more EMMPRIN 45kD than macrophages, confirming in vivo observations.

emmprin45kd		Uni-variat	multi-variat
mmp2 active	p-value	0.027	0.004
	R	-0.180	
mmp2 inactive	p-value	0.045	<0.001
	R	0.170	
mmp9active	p-value	0.056	0.949
	R	-0.160	
mm9inactive	p-value	0.060	0.189
	R	-0.160	
Furin active	p-value	0.057	0.791
	R	0.160	
Furin inactive	p-value	<0.001	0.003
	R	0.290	

Table 4: *Uni-variate and multi-variate regression analysis between EMMPRIN 45kD and furin and MMP levels.*

emmprin58kd		Uni-variat	multi-variat
mmp2 active	p-value	0.721	0.802
	R	-0.030	
mmp2 inactive	p-value	0.001	0.026
	R	-0.270	
mmp9active	p-value	0.022	0.000
	R	0.190	
mm9inactive	p-value	0.727	0.001
	R	-0.030	
Furin active	p-value	0.003	0.002
	R	0.240	
Furin inactive	p-value	0.045	0.160
	R	0.170	

Table 5: *Uni-variate and multi-variate regression analysis between EMMPRIN 58kD and furin and MMP levels.*

DISCUSSION

Atherosclerotic plaque progression is a dynamic process with continuous remodeling of the extracellular matrix. It is thought that MMPs mediate different processes within the atherosclerotic lesion, including matrix degradation, cell infiltration and migration, eventually leading to plaque destabilization and/or rupture. MMPs are secreted as latent pro-enzymes and can be produced by different cell types, including inflammatory cells, fibroblasts and SMCs, and activation is required. Therefore, both the induction of expression as well as the activation of MMPs is important. In the present study we studied the expression levels of two members of the MMP family, MMP-2 and MMP-9, in a large population of atherosclerotic plaques. Moreover, we investigated whether EMMPRIN, an inducer of MMP expression, and furin, an activator of different proproteins, were associated with atherosclerotic plaque characteristics and MMP levels.

MMP expression and plaque phenotype

In the present study the pro- and active MMP-9 levels were associated with more staining for CD68, confirming previous findings that MMP-9 immunostaining mostly co-localizes with macrophages.[5,21] Increased MMP-9 expression has been described in the surrounding of the lipid core of plaques[5] and was related to unstable carotid plaques, indicated by histological evidence of plaque rupture and intraplaque hemorrhage.[22] Our results confirm the association between MMP-9 and an unstable plaque type, since MMP-9 levels were increased when the amount of collagen and SMCs within the lesions was low.

Previously, we observed a more diffuse staining throughout the atherosclerotic plaque for MMP-2, not specifically co-localized with macrophages.[21] In the present study, increased MMP-2 expression levels were associated with the presence of more SMCs. Furthermore, MMP-2 levels were less prevalent when a more atheromatous phenotype was observed, suggesting that MMP-2 expression is associated with a more stable lesion. MMP-2 was not associated with the presence of CD68 staining, although it was previously reported that both MMP-2 and MMP-9 immunoreactivity were more prevalent in expansively remodeled plaques,[21] associated with unstable angina.[23,24] On the other hand, MMP-2 levels did not differ between stable and unstable carotid plaques.[22] Thus, reports on the relation between MMP-2 levels and plaque progression seem conflicting. Regulation of MMP-2 expression differed from MMP-9 which may be due to the constitutive MMP-2 expression by SMCs and macrophages, whereas MMP-9 expression is only present upon stimulation. Our findings suggest that MMP-2 expression is associated with a more stable plaque phenotype.

EMMPRIN and MMP expression

The protein core of EMMPRIN is approximately 27kD, but different forms of EMMPRIN can be produced by different modes of glycosylation (43-66kD),[25] resulting in different molecular weights. EMMPRIN glycosylation is critical for MMP induction

capability,[13,26] but it remains to be elucidated how glycosylation can regulate its induction capacity.

In the present study, EMMPRIN 45kD expression levels were associated with lower macrophage and higher SMC immunoreactivity within the plaque. Furthermore, EMMPRIN 58kD expression was associated with MMP-9 expression that was associated with macrophages. In a previous study, EMMPRIN immunoreactivity was identified in human CD68+ macrophage-rich areas.[15] However, our results imply that EMMPRIN expression is not restricted to macrophages. EMMPRIN 45kD expression levels seem associated with SMC, while EMMPRIN 58kD is expressed by both macrophages and SMCs, but more abundantly by macrophages. This suggests that different cell types produce different amounts of EMMPRIN 45kD and 58kD, resulting in a different MMP repertoire. This was confirmed with EMMPRIN

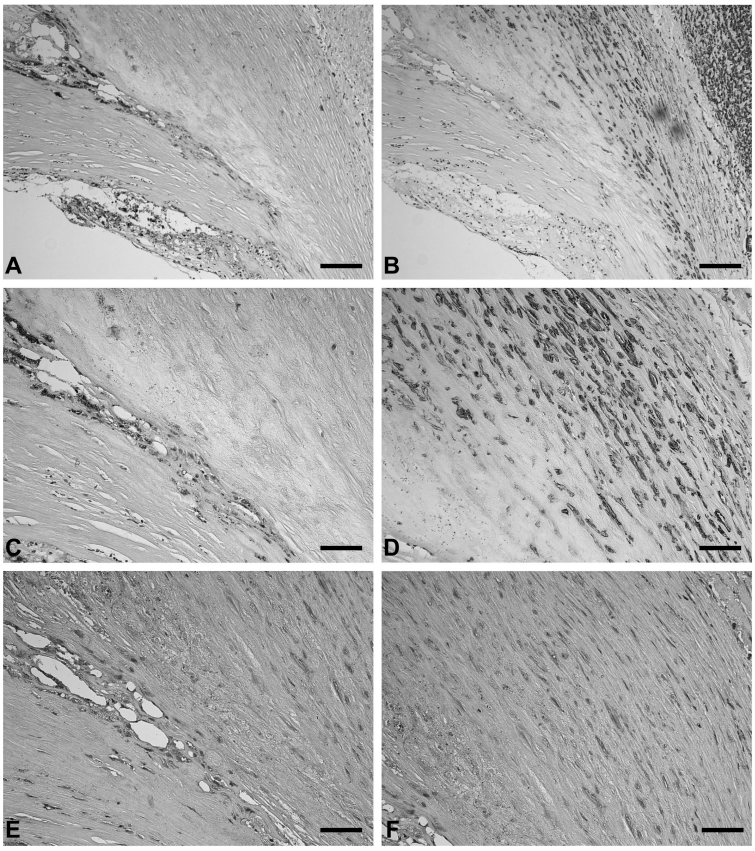

Figure 1: *Immunohistochemical stainings for CD68 (macropages, A+C), alpha actin (smooth muscle cells, B+D), and EMMPRIN (E+F) in atherosclerotic lesions. EMMPRIN immunoreactivity is co-localized with macrophages and smooth muscle cells. (Bar A+B=100μm, Bar C-F=50μm).*

immunoreactivity in macrophages and in SMC-rich areas in atherosclerotic lesions and the presence of more EMMPRIN 58kD under basal culture conditions in macrophages compared to SMCs.

EMMPRIN and furin expression

Furin is not described in atherosclerosis, but as an activator of proproteins, the availability of furin inhibitors, and the involvement of furin in the arterial response to injury,[17] potentially interested. Furin expression was not related to plaque characteristics or MMP expression. Interestingly, a strong correlation was observed between pro-furin and EMMPRIN 45kD levels, and between active furin and EMMPRIN 58kD levels. To our knowledge, no previous reports described an association between EMMPRIN and furin expression levels. Several explanations of our findings could be formulated: first, because EMMPRIN also stimulates the expression of activators of MMPs, like membrane type 1-MMP (MT1-MMP) and MT2-MMP, EMMPRIN expression might regulate furin expression. Furin is described as an activator of several MMPs, like MT1-MMP and stromelysin-1. Secondly, furin might stimulate the expression of EMMPRIN via the activation of one of furin's substrates. It is unlikely that furin will regulate EMMPRIN activity directly, because furin recognizes a specific polybasic sequence motif, not found in the sequence of EMMPRIN. Moreover, glycosylation and not activation regulates EMMPRIN activity. Thirdly, EMMPRIN and furin expressions are up-regulated simultaneously, but not related. Previous reports described that epidermal growth factor (EGF) can stimulate the expression of EMMPRIN[27] but also of furin[28].

Limitations:

The cross-sectional study design does not allow causal inferences. However, the large sample size of human plaques provided important information and demonstrated statistically significant differences among groups. Differences that would have remained unnoted when only smaller numbers of specimen would be investigated. The absence of correlations between histological findings and expression levels could be caused by practical limitations: protein isolation and histological stainings could not be performed on the same segment.

We used zymography to analyze MMP expression and activity, and therefore the in situ MMP activity of our samples could be different because TIMP interactions are lost.

Plaque samples are obtained from a heterogeneous population. Individual patient characteristics could act as a confounder in the investigated relationships. In Athero-Express, however, patients fill in extensive questionnaires. The traditional risk-factors, medication use, and clinically evident cardiovascular disease did not show any confounding effect (not shown)

In summary

We observed in a large scale human atherosclerotic plaque population that MMP-9 levels are associated with an inflammatory atherosclerotic lesion, whereas increased

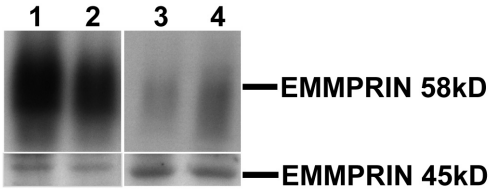

Figure 2: *Western blot analysis of basal EMMPRIN expression in macrophages (lanes 1+2) and smooth muscle cells (lanes 3+4). Both EMMPRIN 45kD and EMMPRIN 58kD can be produced by both cell types. Interestingly, EMMPRIN 58kD levels are higher in macrophages compared to smooth muscle cells. (Western blots are representative for three experiments).*

MMP-2 levels were observed in more stable alpha actin rich atherosclerotic lesions. EMMPRIN 58kD expression levels are not associated with plaque characteristics, whereas EMMPRIN 45kD expression levels are higher in SMC rich lesions. Furthermore, EMMPRIN can be produced by both SMCs and macrophages. Interestingly, the less glycosylated form of EMMPRIN (45kD) is associated to pro-furin and MMP-2 levels, whereas the more glycosylated form of EMMPRIN (58kD) is related to active furin and MMP-9 levels. While, furin expression was associated with EMMPRIN expression, furin expression levels were not related to plaque characteristics or MMP expression.

In conclusion, MMP-9 levels are increased in more unstable plaques, whereas MMP-2 levels are higher in stable lesions. Different glycosylated EMMPRIN forms are associated to other MMP and furin levels. Because differences in EMMPRIN forms are also observed among plaque phenotypes, EMMPRIN may play a role in MMP regulation and the development of atherosclerosis and plaque destabilization.

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8 General discussion

Nowadays, the artery is no longer seen as a rigid tube only responsible for blood supply, nor is the extracellular matrix (ECM), the main constituent of arteries, no longer seen as the static embodiment in which cells reside. The arterial wall actually consists of three layers: (1) The tunica intima, containing connective tissue and endothelial cells, that senses changes in stretch or shear stress, (2) the tunica media, that consists mainly of smooth muscle cells (SMCs) embedded in collagen and proteoglycan fibers and is involved in arterial adaptations to altering pressures, (3) the tunica adventitia, composed primarily of fibroblasts, surrounded by a collagen rich matrix1. Next to the different cell types, the extracellular matrix is also essential for the integrity of artery.

The ECM contain mainly elastin and collagen, that provides a structural framework essential for preserving the shape and structure of the arterial wall. However, it is now well recognized that arteries, and their ECM, continuously adapt in size and composition, which is defined as vascular remodeling. The responses in physiological and pathological arterial remodeling are various and complex, comprising of degradation and synthesis of the ECM, but include also processes like cell proliferation, differentiation and migration. It is suggested that the ECM can store and mobilize growth factors and other signaling molecules, preventing the continuous induction of SMC proliferation. A regulating role of the ECM is demonstrated in elastin null mice, where an uncontrolled proliferation of subendothelial SMCs is observed due to the lack of elastic fibers[2]. In addition, cells are attached to the ECM, and collagen in particular, via adhesion molecules and integrins, necessary for cell attachment. Collagen is the main protein component of the artery[3], and among all the different types of collagen, type I and III are the major fibrillar collagen detectable in arteries[4]. Next to structural integrity, collagen is involved in several cellular processes determining cell phenotype, protein synthesis, and cell migration[5,6]. To preserve the arterial integrity, collagen synthesis and degradation is under tight control in normal physiological processes. However, in different pathological situations, like de novo atherosclerotic plaque development and after arterial injury, collagen synthesis and degradation are out of balance. In this thesis, we studied arterial collagen turnover during physiological and pathological adaptations of the artery and tried to find new targets to intervene in collagen turnover during these processes.

PHYSIOLOGICAL ARTERIAL REMODELING

SUSTAINED FLOW CHANGES

The importance of hemodynamics has been postulated in flow-induced[7,8], de novo atherosclerotic and restenotic arterial remodeling[9]. Furthermore, it was demonstrated that elevations of blood flow had an atheroprotective role and inhibited intimal thickening after vascular injury[10-12]. Variations in regional shear stress also play an important role in the development of atherosclerotic lesions[13,14], moreover, locally

determined shear stress after angioplasty is found to be more predictive for arterial shrinkage than the acute luminal gain[15]. Shear stress is the frictional force at the lumen side tending to displace the endothelium and the inner layers of the artery[16]. After sustained flow changes, the artery starts to adapt to restore apparently preset shear stress levels: a flow increase results in an increase in arterial diameter, whereas a flow decrease results in arterial diameter reduction. The arterial response to chronic flow changes allows studying arterial remodeling without influences of other processes, like neointimal hyperplasia after injury or inflammation during de novo atherosclerosis. This process of arterial remodeling is endothelium and NO-dependent[7,17], and is accomplished through a reorganization of wall constituents[18]. Although these arterial wall constituents change during remodeling, the role of collagen turnover was not studied yet.

Chapter 2, demonstrates that collagen synthesis was increased after both flow increases and flow decreases. The expression of many genes is directly or indirectly affected via shear stress responsive elements in promotor regions, like plasminogen activators and their inhibitor, transforming growth factor (TGF)-β [19], and collagen type IV[20]. Thus collagen synthesis can be regulated directly by shear stress or via TGF-β1, that can stimulate Hsp47 and collagen α1(I) expression in parallel at the translational level[21].

The observed increased collagen synthesis did not result in more collagen deposition in both models. Although, it is not expected that steady state collagen content will be different in healthy arteries, previous reports suggested that increased advential fibrotic response was associated with more arterial constrictive remodeling after injury. Our results demonstrated that arterial remodeling could occur without an increased collagen deposition. There might be a difference between newly synthesized collagen and existing collagen, already present in the matrix[22].

It has been described previously that SMC migration is dependent on the production of de novo collagen to ensure that the ECM is appropriately structured for cell locomotion[23]. Blindt et al[24] described increased Moesin expression in neointimal SMCs and the increased invasive potential of SMCs after Moesin transfection. After flow decrease and increase, Moesin levels were increased during arterial remodeling and associated with increased procollagen expression. This suggests that the increased synthesis of collagen was used for cell migration to increase or decrease arterial dimensions. However, because arterial dimensions were changed, also a modulation of the preexistent collagen matrix is essential.

The expression and activation of the matrix metalloproteinases (MMPs) responsible for matrix degradation but also involved in cell migration, are key events in arterial remodeling. Inhibiting MMP activity blocks expansive aneurysm formation[25,26], but also remodeling after altered blood flow[27] or balloon dilation[28]. Next to increased collagen synthesis, MMP expression was also increased in both models of sustained flow changes, reflected by MT1-MMP mRNA expression and MMP-2 activity (chapter 3). The observed increases in MMP-2 activity were later compared to arterial remodeling, suggesting that another member of the MMP family is responsible for the early response. Next to MMP-2, MT1-MMP and MMP-9 are the most

likely candidates. Increased MT1-MMP expression was present early after flow changes. In MT1-MMP deficient mice a progressive fibrosis is observed in many tissues, probably due to a loss of collagenolytic activity. This might also affect the capability of cells to migrate since cell migration through a collagen rich matrix is, at least partly, MT1-MMP dependent[30,31]. These observations suggest a role for MT1-MMP in matrix degradation and subsequent cell migration. Previously, Sho et al.[29] found an early increase in MMP-9 expression followed by more long term increased MMP-2 expression after flow changes, moreover, a shear-sensitive binding site was found in the MMP-9 promoter[33]. However, a role for MMP-2 cannot be excluded, since arterial blood flow is important in regulating MMP-2 mRNA levels[32]. Experiments that induce flow-related remodeling should be performed in recently developed MMP-2, MMP-9, and MT1-MMP knockout mice to understand the role of these MMPs after flow induced arterial remodeling.

MMPs are synthesized as inactive zymogens and activation is essential for their catalytic capacity. The cell-mediated activation of proMMP-2 is performed by MT1-MMP[34]. Moreover, MT1-MMP possesses a unique insertion of the RRKR amino acid sequence, which is a recognition site for furin. Furin, a member of the proprotein convertases, is a calcium dependent protease and functions as a proteolytic activator of proproteins[35]. The existence of a furin – MT1-MMP – MMP-2 activation cascade was found in vitro, but its presence in the artery was not described. In **chapter 3**, the proprotein convertase – MT1-MMP axis was revealed during flow-induced arterial remodeling. Negishi et al confirmed the potential role of furin in remodeling after flow changes[36]. Furin expression was induced by shear stress, and inhibiting furin activity decreased the formation of mature TGF-β, but the effect on MT1-MMP

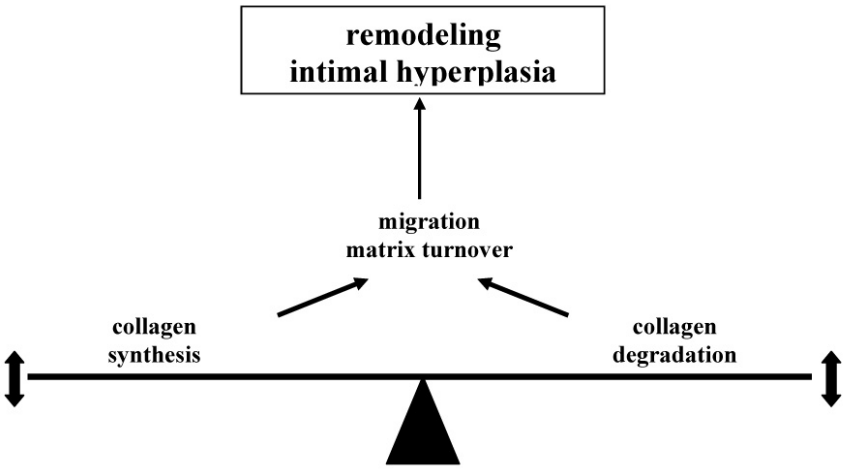

Figure 1: *A tight balance exists between collagen synthesis and degradation. Following arterial injury, this balance is disturbed, leading to constrictive arterial remodeling and neointimal formation, processes in which cell migration and matrix turnover are essential.*

activity was not studied. Interestingly, molecular analysis revealed a positive-feedback loop between furin and TGF-β expression[36]. Because furin activity involves the processing of many substrates it remains to be elucidated which pathway will dominate in arterial remodeling.
The parameters studied in collagen turnover were increased in both models of sustained flow changes, however, the direction of remodeling was different. In both models, the artery tried to restore shear stress levels and in both models collagen turn-over occurred. Especially in atherosclerotic lesions, the direction of arterial remodeling determined luminal size. These data suggests that collagen turn-over itself is not involved in the direction of remodeling; the different responses might be regulated by shear stress dependent factors like eNOS, endothelin or other factors with shear stress responsive elements which needs more investigation.

PATHOLOGICAL ARTERIAL REMODELING

ARTERIAL INJURY

Upon mechanical injury, the artery should have a rapid healing response to preserve the integrity of the vessel wall and to maintain blood supply. However, in its attempt to restore the arterial wall, the artery overreacts, resulting in luminal narrowing, caused by neointimal hyperplasia and arterial shrinkage (figure 1). After arterial injury, several processes are induced, like apoptosis and cell proliferation, ECM production, phenotypic changes and migration of SMCs and adventitial fibroblasts.

Intimal hyperplasia
Upon balloon injury the endothelium is damaged, resulting in loss of endothelial regulation and decreases the availability of vasculoprotective molecules, such as nitric oxide and prostacyclin, ultimately leading to the formation of neointima[37]. It has been shown that transplantation of autologous endothelial progenitor cells (EPCs) effectively reduces neointimal hyperplasia by promoting reendothelialization[38].
In balloon-induced arterial injury, it is thought that upon medial damage, the contractile SMCs in the medial layer dedifferentiate into more synthetic, proliferative, and ECM producing SMCs[39]. These cells migrate and populate the newly formed intimal layer. However, it was shown that lacZ transfected advential fibroblasts could also be detected in the media and intima[40,41] after injury. It has been suggested that fibroblasts, myofibroblasts, and SMCs derive from a common stem cell[42], however due to the lack of specific markers this is still unclear. Six months after bone marrow transplantation, we observed the presence of bone marrow derived cells in the adventitial and endothelial layer of non-injured rat aortas. They contributed to the neointimal layer after in vitro culturing of these arteries (unpublished data). Recently, this was confirmed by Hu et al., showing that a large population of vascular progenitor cells exist in the adventitia and can differentiate into SMCs that contribute to ather-

osclerosis[43]. Thus, in response to arterial injury both adventitial and medial layers use an existing progenitor cell that probably suits the repair process and contributes to neointima formation, however, the lack of specific markers hampers a detailed temporal analysis which cells contribute after arterial injury.

Arterial remodeling

After balloon injury, arterial constrictive remodeling (shrinkage) occurs and accelerates lumen loss. Collagen synthesis has been associated with arterial remodeling[44,45], and, ECM deposition and cell hyperplasia are responsible for adventitial thickening, via collagen deposition. In the normal artery, cellular interactions between SMCs and the ECM is mediated by specific integrins which are required to maintain normal phenotypes. Mechanical injury and the loss of SMC interaction with the ECM will result in increased protein synthesis, including collagen[46-48]. In addition, fibroblasts attached to a strained collagen matrix produce more ECM proteins than cells in a relaxed matrix[49], probably regulated by strain-responsive elements in the promotor regions[49].

After arterial injury (**chapter 4**), procollagen synthesis was increased, reflected by procollagen I mRNA levels and Hsp47 mRNA and protein levels, and an increase in adventitial and medial collagen content (Figure 2). The role of the collagenous adventitia and media in arterial shrinkage is unclear. Several studies describe an increase in collagen fiber content after arterial balloon injury[44,50-54] and found a positive correlation between collagen fiber content and arterial remodeling[51,54] was found. On the other hand, Coats et al[52] showed that in rabbit arteries collagen content is significantly lower in restenotic versus non-restenotic vessels after angioplasty. It was suggested then that the collagen rich adventitial matrix is responsible for the lack of compensatory outward remodeling during neointimal formation[45]. However, it was previously demonstrated that after arterial injury the time course for myofibroblast appearance, ECM deposition, and peak of arterial remodeling do not coincide[45]. We also demonstrated in a flow-induced arterial remodeling model, that a fibrotic response is not necessary for arterial remodeling to occur. **Chapter 4** illustrates that an increased adventitial collagen deposition is present at day 7 after balloon dilation, not increasing in time, while major changes in constrictive arterial remodeling does occur between 7 and 14 days. Although the adventitial collagen thickening is thought to be responsible for the shrinkage of the artery, adventitial and medial collagen content did not co-increase. Interestingly, we observed that arterial shrinkage is associated with an increased expression of lysyloxidase (unpublished data), responsible for the cross-linking of newly synthesized collagen into insoluble collagen fibers. It was demonstrated that even a partial inhibition of the cross-linking processes in young rats, resulted in a destabilization of the aortic wall with increased diameter and reduced strength and stiffness[55]. After balloon dilation, lysyloxidase expression was maximal at 14 days (unpublished data), suggesting that the arterial shrinkage is caused by increased cross-linking of newly formed collagen fibers leading to an increased advential contraction and subsequently arterial shrinkage. Interestingly, TGF-β1 appeared to regulate lysyl oxidase in cultured rat aortic

smooth muscle cells[56].

Procollagen synthesis was maximally increased at 14 days, whereas medial and adventitial collagen content did not increase after the first week, suggesting that the newly synthesized procollagen is not used for increased collagen deposition. We suggested that de novo collagen synthesis was used for cell migration[23] (Figure 2), based on increased Moesin expression and MMP activity, with a maximum at 14 days.

Because of the lack of specific MMP inhibitors, experiments have provided little insight into the role of individual MMPs. In vitro experiments showed that MMPs often share broad substrate specificity. However, knockout models suggest that in vivo MMPs are more specific. For instance MMP-2 and MMP-9 are abundantly present in arterial tissue and are thought to have similar functions based on substrate

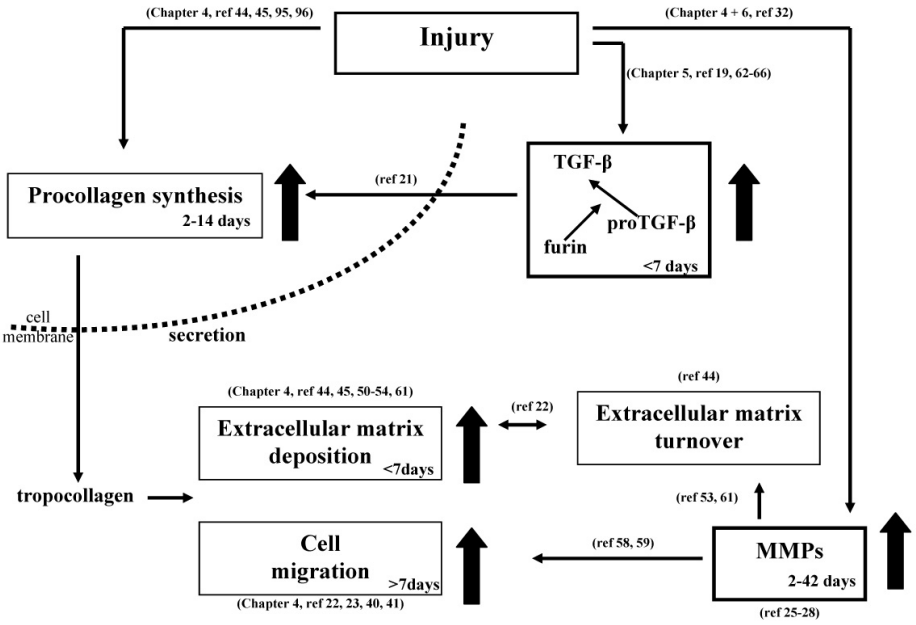

Figure 2: *Summary of the arterial response to injury. After arterial balloon dilation, procollagen synthesis was increased, demonstrated by increased procollagen and Hsp47 expression (2 -14 days). The increased procollagen synthesis resulted in an increase in fiber content (extracellular matrix deposition) within the first week. Collagen content, however, did not increase after the first week, although procollagen synthesis was maximal at 14 days. We suggested that after the first week, synthesized procollagen was used for cell migration and matrix turnover. Increased Moesin expression, a protein associated with smooth muscle cell migration, was observed after the first week and, in addition, EMMPRIN mRNA expression and MMP activity, both involved in cell migration, were maximal at 14 days. Whether the existing collagen matrix was restyled after injury remains to be demonstrated. Collagen synthesis can be regulated by TGF-β expression. We observed an early increase in furin expression and a reduction in TGF-β activity after furin inhibition, suggesting that furin is involved in the arterial response probably via TGF-β activation.*

affinity in vitro, however the in vivo substrates are largely unknown[57]. Recent studies in MMP-2 and MMP-9 defective mice demonstrated that both MMP-2 and MMP-9 were involved in SMC migration after ligation of the mouse carotid artery, but neither reduced SMC migration completely[58,59]. MMP-2 participates in neointimal formation, but not in vessel diameter reduction, via reduction of SMC migration and not due to a reduction in inflammatory cells, whereas MMP-9 has a role in attachment of SMCs to the matrix, facilitating cell attachment to degrade collagen and for traction during cell migration. This is consistent with emerging evidence that cellular proteolytic activity is focalized, limited to the surface of migrating cells by binding to cell surface receptors[60]. The induction of proteolytic activity in vivo is transient, and probably serves to mediate cell detachment and migration through the ECM, and not a widespread degradation of the ECM[61]. It remains to be elucidated, however, whether MMPs degrade mainly mature collagen fibers in the arterial wall or whether they mainly degrade newly synthesized collagen molecules, as suggested in chapter 4, which are not cross-linked and therefore more susceptible to degradation by MMPs (Figure 2).

We suggested the presence of the furin – MT1-MMP – MMP-2 activation cascade during arterial remodeling in chapter 3. In chapter 5, furin involvement was studied in the arterial response to injury. We demonstrated that increased furin expression was observed 2 and 7 days following balloon injury and over-expression of a specific furin inhibitor revealed a reduction in neointimal and adventitial area 14 days after balloon dilation. Several possible substrates of furin have been described, including MT1-MMP and TGF-β, and both were found in the arterial wall after injury. However, based on the temporal expression of active TGF-β levels[62] and MT1-MMP expression, and the effect of the furin inhibitor we suggested that furin is involved in the arterial response to injury probably via activation of TGF-β (Figure 2 + 3). Moreover, the reduced intimal and adventitial areas after furin inhibition are in accordance with the previous studies on TGF-β1 inhibition63-66. As stated previously, TGF-β1 can stimulate Hsp47 and collagen α1(I) expression[21]. Therefore, furin might also be indirectly involved in collagen synthesis (Figure 2). Interestingly, we observed a reduced collagen content (p=0.09), 14 days following balloon injury and furin inhibition (unpublished data).

TGF-β can activate distinct pathways and therefore the role of TGF-β in arterial injury is complex. The Activin receptor-like kinase 5 (ALK5) - Smad 2/3 pathway stimulates collagen production but inhibits cell proliferation, migration and MMP production while the ALK1 - Smad1/5 pathway stimulates cell proliferation, migration and MMP production but inhibits collagen production[67]. These pathways have been extensively studied in endothelial cells, but this might also apply to non-endothelial cells in the artery. Chapter 5 shows that both pathways are activated after balloon dilation, however, since both pathways can influence each other and have different kinetics and threshold levels for TGF-β, local levels will determine which pathway is dominant. Moreover, other protein families like the bone morphogenic proteins (BMP)[68] and activin[69] make the response to injury even more complex since they can also stimulate Smad pathways. Specific ALK 1 and ALK 5 inhibitors

or knockout mice might determine which pathway is dominant in the arterial response to injury.

EMMPRIN has been recently identified as a surface glycoprotein on tumor cells that is able to stimulate the production of different matrix metallopoteinases (MMPs), like MMP-1, MMP-2, MMP-3, MT1-MMP, and MT2-MMP[70-72]. The potential role in the arterial response to injury of EMMPRIN, the extracellular matrix metalloproteinase inducer, is explored in **chapter 6**. After balloon dilation, EMMPRIN protein levels were decreased and associated with increased MMP tissue levels. This decrease in EMMPRIN protein levels was an unexpected observation. We suggested that the potential release of EMMPRIN, as demonstrated in vitro, resulted in a decrease in tissue-associated EMMPRIN. We suggest that EMMPRIN may be released after arterial injury and stimulate adjacent cells to produce MMPs (Figure 3). Later on, stimulation of EMMPRIN mRNA expression was observed, needed to compensate for the released EMMPRIN protein. EMMPRIN mRNA levels were increased and associated with maximum MMP activity. The suggested release of soluble EMMPRIN from arterial lesions into the circulation would result in decreased arterial tissue levels of EMMPRIN and in increased EMMPRIN serum levels, reflecting arterial MMP activity. Serum levels of EMMPRIN were determined, however, no differences between control and atherosclerotic patients were revealed. Therefore, our results do not support the utility of serum EMMPRIN as an indicator of MMP activity in the atherosclerotic lesions.

As discussed in chapter 5, TGF-β signaling in arterial remodeling is known, although results are confusing. This might be due to the existence of two distinct TGF-β receptor pathways. It is not known if both or only one of these pathways is involved in the regulation of arterial remodeling. Because EMMPRIN is essential in MMP expression, and MMP expression is involved in arterial remodeling, EMMPRIN might be regulated by the cross talk between the two TGF-β pathways (Figure 3).

Recently, the Toll-like receptor 4 (TLR4) was found to be involved in arterial remodeling[73]. Toll-like receptors play an essential role in activation of innate immunity, and TLR4 is the receptor for exogenous lipopolysacharide (LPS)[74], endogenous heat shock protein 60[75], and extra domain A of fibronectin (EDA)[76]. In the TLR4 defective mouse no remodeling occurs, furthermore, the artery reacts with a fibrotic adventitial response[77]. TGF-β is involved in the inhibition of the innate immunity response[78], and recently it was shown that Ecsit, an obligatory protein for Toll-like signaling, is also essential in TGF-β signaling[79]. Moreover, TGF-β deficiency in mice leads to extensive inflammation and death, while TLR4 expression was increased[80]. These observations suggest a cross talk between TLF4 and TGF-β, but whether these pathways are related in arterial remodeling and if they can regulate EMMPRIN and MMP expression remains to be elucidated (Figure 3).

PATHOLOGICAL ARTERIAL REMODELING

ATHEROSCLEROSIS

Atherosclerosis is an inflammatory disease characterized by extensive remodeling of the ECM and by the accumulation of lipids and cells in the arterial wall[81,82]. During atherosclerotic lesion progression, the risk for plaque rupture depends on plaque composition rather than plaque size[83,84]. Advanced stable atherosclerotic plaques are characterized by a thick fibrous cap, rich in collagen that depends on matrix deposition, migration, and proliferation of vascular SMCs. Unstable or ruptured plaques contain a thin fibrous cap with a dense inflammatory infiltrate, a large lipid core, plaque erosion, luminal thrombus, and intra-plaque haemorrhage[85]. However, basic research and advances in clinical imaging have underscored the

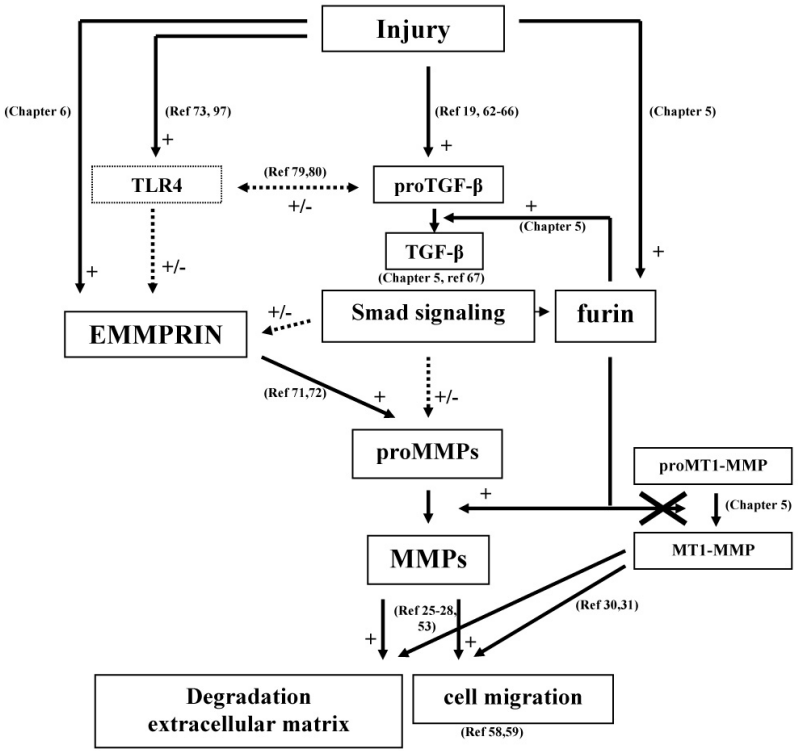

Figure 3: *The potential role of EMMPRIN and furin following arterial injury. Furin is involved in the activation of TGF-β, whereas EMMPRIN stimulates MMP expression. Evidence is emerging that the TGF-β and the Toll-Like Receptor 4 (TLR4) pathways communicate, however, if these pathways can influence EMMPRIN regulation and MMP expression remains to be elucidated.*

realization that while atheroma develops within the intimal layer, the arterial wall undergoes major reshaping to compensate for lumen loss[86,87]. Thus, not only plaque composition is important, but also arterial remodeling will enhance or prevent lumen stenosis and determines clinical outcomes. Expansive remodeling is currently considered one of the characteristic features of vulnerable atherosclerotic plaques. Plaques from patients with unstable angina are associated with expansive remodeling, revealed less fibrous tissue, more inflammatory cells, and larger atheroma compared with plaques from patients suffering from stable angina[88,89]. The presence of macrophages and expression of MMPs have been associated with expansive enlargement in atherosclerotic disease[90], but also with ruptured expansive atheromatous plaques[88,91]. Thus, although outward remodeling will preserve lumen area, its association with unstable plaques is what makes it more deadly. It is difficult to reconcile how a change in vessel area could be causally related with plaque rupture. More likely is the suggestion that remodeling and plaque vulnerability share underlying mechanisms. Recently, it was shown that macrophage content of atherosclerotic lesions play a role in expansive remodeling[92]. They suggested that degradation of the matrix by macrophage derived MMPs allowed the artery to expand in response to a growing lesion, but could eventually weaken the arterial wall. Because atherosclerotic plaque progression is still not well understood, factors that influence plaque composition are of major interest.

Both EMMPRIN and furin could be potential therapeutic targets to inhibit matrix breakdown and subsequent plaque destabilization. However, although their function has been examined in vitro and after arterial injury, validation of expression in relation with a vulnerable plaque is lacking. In **chapter 7**, the relation of EMMPRIN and furin with plaque characteristics is investigated in a large population and their association with MMP activity in the atherosclerotic plaque is described. MMP-9 expression was associated with an inflammatory atherosclerotic lesion, whereas MMP-2 expression increases in more stable lesions. EMMPRIN 58kD expression is not associated with plaque characteristics, whereas EMMPRIN 45kD expression is higher in SMC rich lesions. Interestingly, the less glycosylated form of EMMPRIN (45kD) is related to inactive furin and MMP-2 levels, whereas the more glycosylated form of EMMPRIN (58kD) is related to active furin and MMP-9 expression. While, furin expression was associated with EMMPRIN expression, furin expression was not related to plaque characteristics or MMP expression.

These observations suggest that different cell types (SMCs and macrophages) produce different amounts of EMMPRIN 45kD and 58kD, resulting in a different MMP repertoire in the atherosclerotic plaque, and therefore EMMPRIN may play a role in the development of atherosclerosis and/or plaque destabilization.

CONCLUDING REMARKS AND FUTURE ASPECTS

We observed increased collagen synthesis and increased levels of proteolytic enzymes (MMPs) during arterial remodeling and neointima formation. Furthermore, collagen deposition was not associated with arterial remodeling and intimal formation, suggesting that increased collagen deposition could not be held responsible for constrictive arterial remodeling, but collagen related processes, like increased cross-linking of collagen, might be responsible.

We identified potential new targets for intervention in arterial remodeling and in de novo atherosclerosis. Furin was involved in the arterial response to injury and could be a possible target to inhibit intimal hyperplasia. Furin activated latent TGF-β that can activate distinct pathways (Activin receptor-like kinase pathways), leading to a complex response, influencing cell proliferation, migration, MMP production, and collagen synthesis. Modulation of these pathways might favor the arterial response following balloon dilation.

EMMPRIN expression increased after injury and different EMMPRIN forms were associated with MMP expression in more or less stable plaques, suggesting that EMMPRIN is involved in the arterial response to injury and in plaque progression. Regulation of EMMPRIN expression and the role of different EMMPRIN forms need further studies. The localization on the cell surface, however, makes EMMPRIN an attractive protein for specific interventions during arterial remodeling and in plaque progression. Furthermore, because TGF-β activity can modulate MMP expression, cross-talk of the Activin receptor-like kinase pathways might regulate EMMPRIN expression.

The modulation or inhibition of MMP activity, via intervention on EMMPRIN expression, is not only a possible target for plaque stabilization and inhibition of arterial shrinkage, but also for processes, like collateral formation and heart failure, in which MMPs also play an important role.

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Summary

In the process of atherosclerosis, and during neointimal formation and arterial remodeling, the structure of the arterial wall is changed dramatically. Collagen is one of the major components of the arterial wall, essential for arterial integrity, and therefore increased collagen synthesis and degradation are important processes.

Chapter 1 is a general introduction about the role of collagen turnover in arterial disease and it described collagen synthesis and degradation in particular.
Previous studies suggested a relation between collagen content and arterial remodeling after arterial injury. In **chapter 2**, collagen turnover was studied during flow-induced arterial remodeling. An increase or decreases in flow resulted in a structural arterial increase or decrease, respectively. In both models, collagen synthesis was increased but without a net increase in collagen fiber content, suggesting that the newly synthesized collagen wais not used for increased collagen deposition. We suggested that newly synthesized collagen was used for restructuring of the arterial wall and for cell migration. Increased Moesin expression, associated with the increased potential of smooth muscle cells to migrate, was observed during arterial remodeling. Moreover, the expression of matrix metalloproteinases (MMPs), responsible for collagen degradation but also involved in cell migration, were also increased.
MMPs are synthesized as latent enzymes and they need a cleavage to become proteolytic active. Furin was described to be involved in the activation of some MMPs in vitro. We studied the presence of the furin – MT1-MMP – MMP-2 activation cascade during arterial remodeling in **chapter 3**. Increased expression of all component of this activation cascade was observed during flow induced arterial remodeling.
In the study described in **chapter 4** collagen synthesis, collagen degradation, and collagen fiber content were studied after arterial injury. Arterial shrinkage was associated with increased collagen synthesis and degradation, and Moesin expression, but not with collagen content. These data supported the suggestion that part of the newly synthesized collagen was not used for collagen deposition after arterial injury, but for restyling of the arterial wall and for cell migration. Moreover, we suggested an increased cellular throughput of procollagen molecules following injury.
After the observation of increased furin expression during flow-induced arterial remodeling, the study described in **chapter 5** was designed to study the involvement of furin in the arterial response to injury. Inhibition of furin following balloon dilation resulted in a decrease in adventitial and intimal areas. Moreover, studying temporal expression patterns of potential furin substrates, like MT1-MMP and TGF-β, and the effect of the furin inhibitor, suggested that furin is involved in the arterial response most likely via TGF-β activation.
Increased MMP expression is an important feature during arterial remodeling.

The extracellular matrix metalloproteinase inducer (EMMPRIN), highly glycosylated, is essential for MMP expression in oncological tissue. However, its role in arterial remodeling and atherosclerosis has not been studied. In **chapter 6**, we studied EMMPRIN expression following balloon dilation. EMMPRIN mRNA expression was increased, while tissue-associated EMMPRIN protein was decreased and associated with MMP expression. We suggested that EMMPRIN may be released after arterial injury and stimulate adjacent cells to produce MMPs. Interestingly, EMMPRIN was present in human serum, however, no differences could be observed between control and atherosclerotic patients.

In atherosclerosis, matrix degradation and subsequent plaque rupture are key events. The potential therapeutic targets, furin and EMMPRIN, were studied in human atherosclerotic lesions in **chapter 7**. We observed that furin was not associated with plaque characteristics, but strongly associated with EMMPRIN expression. The less glycosylated EMMPRIN form was associated with MMP-2 expression and increased amount of smooth muscle cells (SMCs), whereas the more glycosylated form of EMMPRIN was associated with MMP-9 expression and increased amount of macrophages. Stable atherosclerotic plaques contain a relatively increased amount of SMCs, while an increased amount of macrophages is associated with plaques that are more vulnerable. This suggests that the different EMMPRIN forms may play a role in MMP regulation and plaque destabilization.

In **chapter 8** the results of the aforementioned studies are discussed.

Samenvatting

Gedurende het proces van aderverkalking, en tijdens neointima formatie en arterieel remodeleren, wordt de structuur van de vaatwand drastisch veranderd. Collageen is een van de meest voorkomende eiwitten in de vaatwand, essentieel voor het behouden van de integriteit van het vat. Daardoor zijn de verhoogde productie en afbraak van collageen belangrijke processen.

Hoofdstuk 1 is een algemene introductie over de rol van collageen omzetting in arteriële ziektes en het beschrijft de collageen synthese en afbraak meer in detail.
Verschillende studies suggereerden een relatie tussen de hoeveelheid collageen en de mate van arterieel remodeleren na beschadiging van de arterie. In **hoofdstuk 2** werd de collageen omzetting bestudeerd tijdens arterieel remodeleren na chronische bloed stroom toe- en afnames, wat resulteerde in respectievelijk structurele toe- of afnames in arteriële diameters. De collageen productie was in beide modellen toegenomen, maar zonder een netto toename in de hoeveelheid collageen vezels, dit suggereerde dat het nieuw geproduceerde collageen niet gebruikt werd voor een toename in collageen afzetting. We stelden voor dat het nieuw geproduceerde collageen gebruikt werd om de vaatwand te herstructureren en voor de migratie van cellen. De verhoogde productie van Moesin, dat geassocieerd is met een verhoogde potentie van gladde spier cellen om te migreren, werd gevonden tijdens het remodeleren van de arterie. Bovendien was ook de productie van de matrix metalloproteinases (MMPs), die verantwoordelijk zijn voor de afbraak van collageen, maar ook betrokken zijn bij de migratie van cellen, verhoogd.
MMPs worden geproduceerd als inactieve enzymen en ze hebben een knip nodig om proteolytisch actief te geraken. Furin was beschreven betrokken te zijn bij de activatie van sommige MMPs in vitro. Wij bestudeerden de aanwezigheid van de furin –MT1-MMP- MMP-2 activatie cascade tijdens arterieel remodeleren in **hoofdstuk 3**. Een verhoogde aanwezigheid van alle componenten van deze cascade werd gevonden gedurende bloed stroom geïnduceerd remodeleren.
In de studie, beschreven in **hoofdstuk 4**, werd de collageen productie, de collageen afbraak en de hoeveelheid collageen vezels bestudeert na een beschadiging van de arterie. Het krimpen van het vat was geassocieerd met een toename in de collageen productie en afbraak en met een toename in Moesin expressie. De hoeveelheid collageen vezels in de vaatwand nam echter niet toe tijdens het krimpen van het vat. Deze gegevens ondersteunen het voorstel dat een deel van de geproduceerde collageen na een beschadiging aan de arterie niet gebruikt wordt voor een toename in collageen afzetting, maar voor het restylen van de vaatwand en voor de migratie van cellen. We suggereren bovendien dat na een beschadiging de cellulaire verwerkingcapaciteit van pro-collageen toeneemt.

Na de observatie van een verhoogde productie van furin gedurende bloed stroom geïnduceerd vaatwand remodeleren, werd de studie opgezet, beschreven in **hoofdstuk 5**, om de betrokkenheid van furin te bestuderen in de reactie op een beschadiging van het vat. Remming van furin na ballon dilatatie resulteerde in een afname van adventitia en intima oppervlaktes. Het bestuderen van de tijdelijke expressie patronen van potentiële furin substraten, zoals MT1-MMP and TGF-β, en het effect van de furin remmer, suggereerde bovendien dat furin waarschijnlijk betrokken is in de arteriële reactie via de activatie van TGF-β.
De verhoogde productie van MMPs is een belangrijk aspect gedurende arterieel remodeleren. De extracellular matrix metalloproteinase inducer (EMMPRIN), een sterk geglycosyleerd eiwit, is essentieel voor de MMP productie in oncologische weefsels, maar zijn rol in arterieel remodeleren en aderverkalking is niet bestudeerd. In **hoofdstuk 6** hebben we EMMPRIN productie bestudeerd na ballon dilatatie. De expressie van EMMPRIN mRNA was toegenomen, terwijl het weefsel gebonden EMMPRIN eiwit afnam, en was geassocieerd met MMP productie. We suggereerden dat EMMPRIN vrij komt na arteriële beschadiging en naburige cellen stimuleert om MMPs te produceren. Belangwekkend was de aanwezigheid van EMMPRIN in het serum, helaas konden er geen verschillen worden gevonden tussen controle en atherosclerotische patiënten.
Tijdens aderverkalking zijn de afbraak van matrix en de daaropvolgende plaque ruptuur toonaangevende gebeurtenissen. De potentiële therapeutische doelen, furin en EMMPRIN, werden bestudeerd in humane atherosclerotische plaques in **hoofdstuk 7**. We vonden dat furin niet geassocieerd was met plaque karakteristieken, maar wel een sterke relatie had met EMMPRIN hoeveelheden. De minder geglycosyleerde EMMPRIN vorm is geassocieerd met MMP-2 hoeveelheden en met een toename in gladde spier cel kleuring, terwijl de sterker geglycosyleerde EMMPRIN geassocieerd is met MMP-9 hoeveelheden en met en toename in het aantal macrofagen. Meer stabielere plaques bevatten een relatief verhoogde hoeveelheid gladde spier cellen, terwijl een verhoogd aantal macrofagen geassocieerd is met een meer zwakkere plaque. Dit suggereert dat verschillende EMMPRIN vormen een rol spelen in MMP regulatie en plaque destabilisatie.
Hoofdstuk 8 is een algemene discussie over de resultaten van voorgaande studies.

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CURRICULUM VITAE

Joost Sluijter werd geboren op 14 april 1977 te 's-Hertogenbosch. Het V.W.O. diploma behaalde hij in 1995 aan het Orduynen College te 's-Hertogenbosch. Daaropvolgend begon hij in september 1995 met de studie Medische Biologie aan de Universiteit Utrecht, waarvan het propedeuse werd behaald in 1997. Tijdens het doctoraal werden twee onderzoeksstages gelopen in het Universitair Medisch Centrum Utrecht. De hoofdvakstage werd verricht bij de vakgroep Experimentele Cardiologie onder leiding van Dr. D.P.V. de Kleijn en Prof. Dr. C. Borst. De bijvakstage werd verricht bij de vakgroep Interne Geneeskunde onder leiding van Dr. E. Stroes en Prof. Dr. T. Rabelink. Het doctoraalexamen Medische Biologie werd behaald in 2000. Daarna begon hij in mei 2000 in het Laboratorium voor Experimentele Cardiologie van het Universitair Medisch Centrum Utrecht met het onderzoek dat uiteindelijk heeft geresulteerd tot dit proefschrift.
Vanaf mei 2004 is hij werkzaam als postdoc onderzoeker bij het Laboratorium voor Experimentele Cardiologie van het Universitair Medisch Centrum Utrecht, waar hij vanaf september 2004 zijn werkzaamheden zal verrichten aan het Indiana University-Perdue University te Indianapolis onder leiding van Prof. Dr. Z.S. Galis.

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