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

Joost P.G. Sluijter\textsuperscript{a,b}, Mirjam B. Smeets\textsuperscript{a,b}, Evelyn Velema\textsuperscript{a}, Gerard Pasterkamp\textsuperscript{a,b}, Dominique P.V. de Kleijn\textsuperscript{a,b,*}

\textsuperscript{a}Experimental Cardiology Laboratory, University Medical Center, Utrecht, The Netherlands
\textsuperscript{b}Interuniversity Cardiology Institute of the Netherlands (ICIN), Utrecht, The Netherlands

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Abstract

Objective: In the arterial response to injury, collagen breakdown has been 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 following 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 heat shock protein 47 (Hsp47) expression, increased 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. Collagen content in media and adventitia only increased between days 2 and 7 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. This suggests that after 1 week, collagen turnover is used for other processes like cell migration and arterial remodeling.

\textsuperscript{*} Corresponding author. Experimental Cardiology Laboratory, University Medical Center, Heidelberglaan 100, Room G02-523, 3584 CX Utrecht, The Netherlands. Tel: +31-30-2507155; fax: +31-30-2522693. E-mail address: d.dekleijn@hli.azu.nl (D.P.V. de Kleijn).

1. Introduction

Arterial lumen loss after injury is determined 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 et al. [6,7] showed an increase in collagen synthesis and matrix metalloproteinase activity shortly after a second injury.

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, bindsprocollagen 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-pro-peptides 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 with the increase in collagen fiber content only in the first week after balloon injury. The continued increase of collagen synthesis and breakdown after 1 week is associated with remodeling and cell migration.

2. Materials and methods

2.1. Animals

Twenty-five New Zealand White rabbits (Broekman Charles River, 3–3.5 kg) were anesthetized by methadone (0.15 ml) and vetranal (0.15 ml) followed by etomidate (1 mg/kg) and ventilation with N2O/O2/0.6% 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 25 rabbits were balloon dilated unilaterally with a 3.0 mm balloon three times for 30 s. We measured arterial lumen diameter at termination and post-dilation by angiography, with the use of nitroglycerin (200 nM) 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 sum mating the angiographical lumen area and the histological intima area. The relative IEL area change and intima area are calculated by dividing it with the IEL area (post-dilation).

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

2.2. RNA and protein extraction

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

2.3. Cell and tissue culture

In vitro: A cell line of vascular smooth muscle cells (vSMC; 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.

2.4. Quantitative RT-PCR

Rabbit Hsp47 (Forward: 5'-aacatcgaggctggct-3'; Reverse: 5'-aactcaaatcaatacctc-3'); rabbit collagen type I (Forward: 5'-tgccatatgcttcg-3'; Reverse 5'-caatacctggaatgcc-3') and ribosomal 18S (Forward:5'-tcaccaagggaaactca-3'; Reverse:5'-aataaatggctcacaac-3') primers were designed using the Prime program at CMBI (Nijmegen).

First-strand cDNAs were produced using 200 ng RNA and Ready-To-Go You-Prime First-Strand Beads (Amer sham 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, 1 × 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 94 °C followed by 40 cycles of: 30 s at 94 °C, 30 s at 62 °C (Hsp47), 56 °C (collagen I) or at 50 °C (18S) and 1 min at 72 °C. 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 or a melting curve after the PCR. Data are presented as the ratio between the dilated and control arteries.
2.5. 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), 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), 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 CaCl₂, 50 mM Tris–HCl (pH 7.5) 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 detected in the artery at all time points (not shown). No neointima was detected in contralateral uninjured arteries. Only a few macrophages were present at 28 days (16.7% at day 28)(Fig. 1). No neointima was detected in contralateral uninjured arteries. Only a few macrophages were present at 28 days (16.7% at day 28)(Fig. 1).

3. Results

3.1. 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, which was significant at day 14 (−17.7%, p = 0.05). At day 28, the IEL decreased by −25.5% (p = 0.01) (Fig. 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) (Fig. 1). No neointima was detected in contralateral uninjured arteries. Only a few macrophages were detected in the artery at all time points (not shown).

3.2. 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) (Fig. 2A), 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 collagen 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.

2.8. 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.
reached significance at day 7 ($p=0.008$) and day 14 ($p=0.002$), and declined at day 28. Western blotting (Fig. 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 (Fig. 3A,C), the adventitial layer (Fig. 3A,D) and some staining was found in the media (Fig. 3A). No staining was found using the isotype control (Fig. 3B). Cells positive for Hsp47, in both the intima and adventitia,
stained positive for vimentin, thus being fibroblast-like cells (data not shown).

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 ($p = 0.001$; Fig. 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 days 2, 7 and 28. Only at day 14 there was a small but significant increase (1.6-fold, $p = 0.004$, Fig. 4B) compared to control arteries.

3.3. 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 days 14 and 28 (Fig. 4C). Also in the media, a significant increase in collagen content at day 7 ($p = 0.013$; Fig. 4D) was found compared to the adventitial collagen content of contralateral uninjured arteries. Similar to the adventitia, medial collagen content did not increase further at 14 and 28 days. The amount of collagen content in the intima (Fig. 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.

3.4. 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 (Fig. 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 (Fig. 5A, lane 1) and by collagen I pAb (Fig. 5A, lane 3) had the same molecular weight. As expected, in the collagenase-treated samples, the procollagen bands were degraded (Fig. 5A, lanes 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 (Fig. 5B).

Fig. 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 time point. (Data are presented as the ratio operated/control mean ± S.E.M.; femoral and iliac arteries of each survival time are pooled. $N = 6–8$ rabbits per time point, *$p < 0.05$).
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 (Fig. 5C, lanes 1 and 2, y).

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 (Fig. 5C, lane 4, Δ) and even more Hsp47 expression after injury and TGF-β1 stimulation (Fig. 5C, lane 5, Δ) compared to uninjured control aortic rings (Fig. 5C, lane 3, Δ). This was accompanied by increasing levels of procollagen I bound to Hsp47 (Fig. 5C, bottom panel, †) but not by the free procollagen I (Fig. 5C, top panel, ††), confirming the observed results in vivo.

3.5. 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 were significantly increased in the balloon dilated arteries after 7 days (p=0.01) and reaching a maximum at 14 days (p=0.003) (Fig. 6A). Active MMP-
2 levels were increased compared to control levels at 2 days \((p=0.004)\) after balloon dilation and reached its maximum also at 14 days \((p=0.002)\) (Fig. 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 \((p=0.04)\). (Fig. 6C).

### 4. Discussion

Mechanisms of arterial remodeling and neointima formation as a response to injury are still unclear. Until now, in vivo studies have focused 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.

Following balloon dilation, luminal narrowing is the result of two processes: arterial shrinkage and intimal hyperplasia (Fig. 1) [3,25,26]. In our model, intimal hyperplasia was observed from day 7, increasing in size at 14 and 28 days which is in accordance with previous reports by Rasmussen et al. [27] and Barron et al. [28]. Also, the time sequence of 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].

After balloon dilation, we observed increased collagen mRNA levels, accompanied by increased Hsp47 mRNA and protein levels with a maximum increase at 14 days (Figs. 2A,B and 4A). Karim et al. [31] 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. 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. [34] described no adventitial Hsp47 expression after arterial injury of the rat. 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 expression 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 days 7, 14 and 28 after balloon dilation (Fig. 4C,D). However, a maximal increase in collagen content was reached at day 7 in the media and adventitia, and at that time point, the relative fiber content of the intima is very low. Therefore, we think that the peak in collagen expression levels found at 14 days cannot be used for collagen fiber formation alone.

Another explanation can be deduced from the matrix metalloproteinases MMP-2 and MMP-1 levels (Fig. 6) which were maximal at 14 days compared to control arteries. 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 days 7 and 14 (Fig. 1) when collagen mRNA levels are maximal. This association suggests a role for collagen turnover 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 (Fig. 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 (Fig. 2B) and Hsp47-bound procollagen I [21,23] (Fig. 5B), increased 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 (Fig. 5C, lanes 1 and 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 (Fig. 5B). Although Hsp47 and Hsp47-bound procollagen levels increased, we only found a small increase in procollagen I levels not bound to Hsp47, at day 14 and not at day 7 (Fig. 4B).

Hypothetically, these results suggest that arterial adaptation to injury initiates the following collagen synthesis pathway (Fig. 7): In the normal control situation, there is a constant throughput (small arrow) of procollagen with normal (N) levels intra- and extracellularly. 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 in collagen synthesis is too large (Fig. 4B, day 14).

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 did not examine 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 3 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, our results support the data of Strauss et al. [6,7] and Karim et al. [31]. In this study, we analyzed the arterial

![Diagram](https://example.com/figure7.png)

**Fig. 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.
response to injury at different expression levels: procollagen mRNA, procollagen protein, collagen content and MMP activity. We associated these levels with arterial morphometrical changes and included the adventitial layer in our analysis to better understand the mechanisms playing 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 relationship 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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