Arterial haptoglobin

expression, regulation and function

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Arterial haptoglobin: expression, regulation and function
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(met een samenvatting in het Nederlands)

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Exhilaration - the feeling you get just after a great idea hits you, and before you realize what's wrong with it.





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General introduction

Chapter I

General introduction

Arterial restructuring is an important limiting factor for the success rate of balloon angioplasty. In recent years, a large number of articles point to an important role of collagen turnover in arterial restructuring. However, our knowledge about the precise regulators of collagen turnover remains incomplete. In order to develop specific inhibitors of arterial restructuring, it is crucial to obtain a better understanding of the molecular pathways involved in collagen turnover and to elucidate the function and regulation of proteins involved in collagen turnover. In this thesis, we used subtraction PCR to identify new candidate proteins that are involved in arterial restructuring. One of the identified proteins is the acute phase protein haptoglobin which is normally produced in the liver. Arterial haptoglobin expression is potentially very interesting as haptoglobin has been associated with aneurysm formation, angiogenesis and controlling cathepsin and elastase activity. In this thesis, the expression, regulation and function of arterial haptoglobin is investigated and related to arterial restructuring.

Arterial restructuring

Atherosclerosis is a multifactorial disease, being recognized as the number one cause of mortality in the western world. The formation of an atherosclerotic plaque leads, together with remodeling of the artery, to luminal shrinkage and eventually to arterial obstruction. A widely used treatment for occlusive atherosclerotic lesions is percutaneous transluminal angioplasty (PTA, balloon angioplasty), where an inflated balloon compresses the plaque into the arterial wall. However, restructuring of the arterial wall (restenosis) occurs in 30-50% of the cases[1-2], thereby nullifying the benefit of balloon angioplasty. Nowadays, 70% of the coronary revascularizations involve the use of a stent, a flexible metal tube that mechanically prevents arterial shrinkage.

Arterial restructuring after balloon angioplasty is determined by two different processes: geometric remodeling of the artery and neointima formation. Geometric remodeling is defined as a structural change in total arterial circumference. Enlargement of the artery compensates for lumen loss due to neointima formation while shrinkage of the artery (constrictive remodeling) accelerates lumen narrowing. Neointima is formed in response to arterial injury and consists of cell migration and extracellular matrix accumulation. Both human and animal studies demonstrated that constrictive remodeling is the major determinant of arterial restructuring[3-4] and can explain up to 70% of the observed lumen loss.

Arterial restructuring following balloon angioplasty is a complex process that involves cell proliferation, cell migration and remodeling of the extracellular matrix. Although the extracellular matrix is traditionally seen as an inert scaffold, extracellular matrix turnover is a prerequisite for arterial restructuring. Recent studies have demonstrated that turnover of the extracellular matrix regulates many cellular processes that are also observed after balloon angioplasty[5]. In general, there is enhanced collagen synthesis and degradation in the arterial wall within the first week after balloon angioplasty, followed by a gradual accumulation of collagen.

Arterial restructuring and collagen synthesis

Since collagen type I and III are the most abundant extracellular matrix proteins in arteries and play an essential role in maintaining the structural integrity of the artery[6], the effects of balloon angioplasty on the turnover of these two types of collagen have been thoroughly investigated in various animal models[7-8]. Synthesis of collagen type I and III is induced early after arterial injury and mainly localized in the adventitial layer and newly formed neointima[9-10]. This increase in collagen synthesis is followed by a gradual accumulation of collagen, coinciding with an increase in cross-sectional vessel area. The observed delay in collagen accumulation is probably accounted for by enhanced collagen

degradation in the same time-period as increased collagen synthesis[11].

Although it is generally believed that intact collagen fibers form a structural barrier for cells to prevent cell migration[12], there is increasing evidence that active collagen synthesis plays an important role during cell migration. Increases in collagen synthesis coincide with cell migration towards the neointima and intact collagen can promote cell migration by functioning as a chemotactic factor[13-14] or by providing the correct substrate for cell migration[15]. In addition, inhibition of de novo collagen synthesis impairs cell migration[16], implying that only newly synthesized collagen is able to serve as a suitable substrate for cell migration.

Changes in arterial collagen content after balloon angioplasty may have pronounced effects on the arterial remodeling response. Collagen content increases after balloon angioplasty and the accumulation of collagen has been associated with constrictive remodeling and the severity of restenosis after balloon angioplasty[17]. As collagen type I provides structural strength to the artery, the accumulation of collagen type I in the adventitial layer suggests a role in the resistance of the artery to remodeling. This is supported by the association between decreased adventitial collagen content after MMP inhibition and the observed inhibition of constrictive remodeling in favor of arterial enlargement[18-19].

Another mechanism through which collagen accumulation may influence constrictive remodeling involves the crosslinking of newly synthesized collagen by lysyl oxidase into compact collagen fibers. Several studies demonstrated that lysyl oxidase is involved in the contraction of collagen matrices[20-21]. Moreover, reduced lysyl oxidase activity is associated with aortic aneurysm development[22-23] whereas increased lysyl oxidase activity has been implicated in the pathogenesis of atherosclerosis[24].

Arterial restructuring and collagen degradation

Degradation of collagen requires the action of matrix metalloproteinases (MMPs), a family of zinc-containing endopeptidases that collectively are capable of degrading all components of the extracellular matrix. With the exception of stromelysin-3[25], MMPs are secreted as inactive zymogens that are cleaved into mature active enzymes. MMP activity is strictly regulated at several levels including gene transcription, zymogen activation and binding to tissue inhibitors of MMPs (TIMPs).

Increased MMP activity influences both neointima formation and constrictive remodeling after balloon angioplasty. Collagen degradation after arterial injury induces the expression of many genes that are associated with restenosis and normally suppressed by polymerized collagen[26]. Expression and activation of MMPs is increased early after balloon angioplasty with peak levels within the first week [11, 27-29] and accompanied by increased expression levels of TIMPs[29]. Increased MMP activity is associated with the development of arterial restructuring [27, 30-31] whereas MMP inhibition prevents constrictive remodeling and subsequent luminal narrowing[18, 32-33].

The relation between MMPs and neointima formation has been investigated extensively and mainly linked to increased cell migration after arterial injury. In vitro studies have demonstrated that cell migration depends on active collagen synthesis[16] as well as collagen degradation[12, 34-35]. Cell surface localization of several MMPs allows highly localized and tightly controlled proteolysis of the extracellular matrix, thereby enabling cell migration[35-36]. Furthermore, in vivo studies have shown an association of active MMP-2[27, 30] and MMP-9[37] with smooth muscle cell migration into the intima and that inhibition of MMPs results in a nearly complete inhibition of smooth muscle cell migration into the intima. The initial effects of MMP inhibitors on neointima formation looked promising at early timepoints after arterial injury and were related to decreased migration of smooth muscle cell towards the neointima. However, increased cell proliferation in the neointima was responsible for a catch-up in neointimal area at later timepoints[38-39]. Thus, the beneficial effect of MMP inhibition on neointima formation seems only temporary.

Arterial remodeling has been compared to scar contraction which also requires restructuring and degradation of the extracellular matrix. Several studies have linked the expression and activity of several MMPs to either arterial enlargement[40-41] or shrinkage[33] of the artery. In contrast to the large number of data that describe the role of MMPs in neointima formation, data regarding the involvement of MMPs in arterial remodeling are limited. Increased MMP-9 activity is associated with arterial enlargement during aneurysm formation[42] and after arterial injury[37]. Moreover, aneurysm formation can be blocked by MMP inhibitors[43] or TIMP-1 overexpression[44] and constrictive remodeling after balloon angio-plasty can be reduced by MMP inhibition[18, 33].

Inhibition of collagen turnover

Due to the essential role of collagen turnover in arterial restructuring, inhibition of collagen synthesis or degradation may have beneficial effects on the success rate of balloon angioplasty. Collagen synthesis can be blocked at several levels including gene expression, triple helix stability and fiber formation. Although some studies provided promising results for the prevention of arterial restructuring[45-47], other studies demonstrated that collagen synthesis inhibition is not always beneficial[48].

Inhibition of collagen degradation has become the main focus of attention to prevent arterial restructuring. MMP activity can be blocked in various ways, either by reduction of the levels of active MMP or by direct inhibition of MMP activity. In the past, most studies used synthetic broad spectrum MMP inhibitors that bind to the zinc atom at the active center, thereby inactivating the enzyme. Recently, also the effect of TIMP overexpression has been investigated[49-50] which resulted in the reduction of neointimal lesions after arterial injury. However, similar to the synthetic MMP inhibitors, TIMPs are multifunctional and have diverse activities that are not only related to degradation of the extracellular matrix[51]. In general, MMP inhibition results in less cell migration towards the intima and decreased collagen accumulation, thereby attenuating neointima formation and arterial remodeling.

Subtraction PCR

Despite the overwhelming evidence that collagen turnover is essential during arterial restructuring after balloon angioplasty, inhibition of collagen turnover has provided little insight into the role of individual proteins as most studies used broad spectrum inhibitors that exert their effects on multiple proteins. Thus, our knowledge about the precise regulators of collagen synthesis and degradation remains incomplete and makes it essential to elucidate the function and regulation of individual proteins involved in the collagen turnover pathways.

To facilitate the discovery of novel, differentially expressed genes between different tissues of interest, a variety of methods have been developed including differential display PCR[52], RNA fingerprinting[53], suppressive subtraction PCR[54] and DNA microarrays[55]. Although most researchers focus on DNA microarrays, this technique has several disadvantages. The major disadvantage is that microarrays generally cover only the most common model systems like human, mice and yeast. Thus, DNA microarrays are not suitable for the identification of novel, differentially expressed genes in animal models such as the rabbit or pig. In addition, more sensitive techniques like the subtraction PCR allow the detection of sequences that have low expression levels and will not be detected using a microarray.

In this thesis, subtraction PCR is used as an open approach to identify novel, differentially expressed genes involved in arterial restructuring. The subtraction PCR is based on the selective amplification of genes that are expressed in one mRNA population but reduced or absent in another. The method has proven to effectively enrich differentially expressed genes without prior knowledge about gene expression patterns or functional and biochemical protein characteristics.

Theoretically, balloon dilated arteries provide the best material for the identification of genes involved in arterial restructuring. There are, however, some practical disadvantages about the use of balloon dilated arteries in a subtraction PCR. Arterial restructuring is a multifactorial process that is determined by neointima forma-

tion as well as geometric remodeling, making it very difficult to determine whether newly identified proteins are involved in only one of these processes or in both. Compared to balloon dilated arteries, the rabbit flow ligation model represents a simplified model to focus on arterial remodeling without the interference of neointima formation. Hence, we used the rabbit flow ligation model as the basis for a subtraction PCR to identify novel, differentially expressed genes that are involved in arterial remodeling and collagen turnover.

Identification of novel genes involved in arterial restructuring

The subtraction PCR was performed with cDNA from shrunken and control rabbit arteries (respec-tively flow decrease and normal flow), resulting in a subtraction library of approximately 500 clones that were overexpressed in the shrunken arteries. Ninety-six of these clones were randomly selected for verification using dot blot analysis (figure 1). Initial screening indicated that 30 clones were potentially differentially expressed although sequence analysis revealed that several of the clones represented the same gene. Thus, from the original 96 clones, 11 genes with known sequences and 9 genes with unknown sequences were identified as being differentially expressed (table 1).

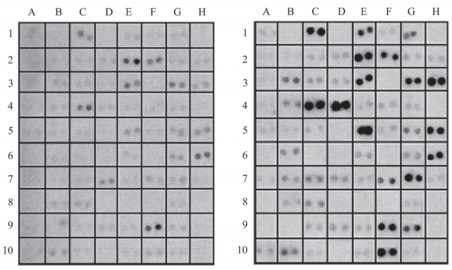

Figure 1. Dot blot analysis of clones generated by subtraction PCR
Two identical dot blots were made by transfer of cDNA from clones to nitrocellulose membranes. The dot blots were hybridized with a 32^{P} labeled probe of the control minus flow decrease library (left panel) and the flow decrease minus control library (right panel). Clones with at least a 2-fold difference in expression between the two different probes (for example C1, D4, G7 and F10) were selected for further analysis.

Table 1. Clones identified using subtraction PCR

clone	sequence	clone	sequence
R1002	hyaluronan	R1076	EST
R1003	elongation factor 1	R1078	EST
R1004	EST	R1082	EST
R1010	hevin-like protein	R1084	EST
R1019	FHL-1	R2005	EST
R1030	cytochrome oxidase	R2014	fibronectin
R1033	EST	R2018	alpha SMC actin
R1045	haptoglobin	R2027	gamma SMC actin
R1054	EST	R2025	EST
R1070	lumican	R2041	tenascin

Although genes with unknown sequences are potentially very interesting, the lack of, for example, antibodies and knockout mice makes it very difficult to identify functional relations between these genes and arterial remodeling. Therefore, we mainly focussed on genes with known sequences. These genes can be classified into two groups: 1) genes previously described to be expressed in arteries (e.g. fibronectin[56] and tenascin[57]) and 2) genes that were not known to be expressed in arteries (e.g. FHL-1 and haptoglobin). Differential expression of candidate genes was further verified by Northern blot analysis, quantitative PCR and western blotting.
One of the identified genes using the subtraction PCR was haptoglobin (figure 2). Haptoglobin is potentially very interesting since it was not known to be expressed in the arterial wall. In addition, previous studies implied a role for haptoglobin in atherosclerosis or related processes like angiogenesis, extracellular matrix degradation and modulation of the immune system. Therefore, we focussed on haptoglobin and investi-

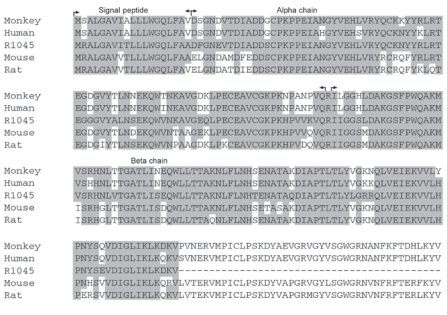

Figure 2. Multiple alignment of rabbit haptoglobin (clone R1045) with monkey, human, mouse and rat haptoglobin
Grey boxes indicate identity with R1045.

gated arterial haptoglobin expression, regulation and function during arterial remodeling.

Haptoglobin synthesis

Until recently, the liver was thought to be the major site of haptoglobin synthesis. However, haptoglobin is also expressed at basal levels in extrahepatic tissues and expression can be induced in various tissues by lipopolysaccharide (LPS)[58-59]. Haptoglobin expression is mainly regulated at the transcriptional level by interleukin-6 (IL-6) although expression of haptoglobin can also be induced by IL-1, tumor necrosis factor (TNF) and glucocorticoids[60].

Haptoglobin is synthesized as a single polypeptide (figure 3) that contains both the non-glycosylated α-chain and the glycosylated β-chain of haptoglobin[61]. Posttranslational cleavage results in the formation of single α- and β-chains that are subsequently linked by disulfide bridges to form mature haptoglobin ($\alpha 2\beta 2$)[62].

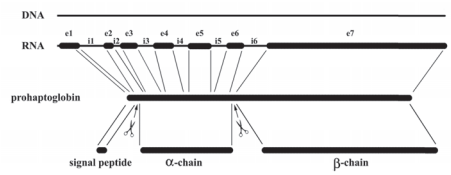

Figure 3. Schematic representation of the haptoglobin gene, RNA and protein
e1-e7 indicate exons, i1-i6 indicate introns.

Haptoglobin function

According to the amino acid sequence, haptoglobin is homologous to serine proteases of the chymotrypsinogen family[63]. This homology is further supported by similarities in posttranslational processing between haptoglobin and tissue-type plasminogen activator[64]. However, haptoglobin contains a small insertion in the region corresponding to the methionyl loop of serine proteases and a replace-ment of two of the active-site amino acids typical of the serine proteases[63]. Together, these alterations result in homologous proteins with different biological functions.

Haptoglobin has a strong non-covalent binding capacity for hemoglobin and the formation of haptoglobin-hemoglobin complexes is believed to be important for the hepatic clearance of hemoglobin from the serum. The condition of ahaptoglobinaemia is, however, clinically silent[65]. Furthermore, haptoglobin knockout mice are viable and have no impaired hemoglobin clearance[66], indicating that hepatic clearance of hemoglobin is not an essential function of haptoglobin. The physiological role of haptoglobin-hemoglobin complexes is, however, not restricted to hemoglobin clearance. The antibacterial properties of haptoglobin depend on capturing hemoglobin-bound iron[67] and the antioxidative role of haptoglobin is related to its binding to hemoglobin which can act as a strong pro-oxidant[68-69]. Functions of haptoglobin that are not related to the binding of hemoglobin include modulation of the immune system[70-71], stimulation of angiogenesis[72] and inhibition of cathepsin B[73] and elastase activity[74].

Haptoglobin polymorphism

In humans, the haptoglobin gene is characterized by a polymorphism with two common alleles for the α-chain (Hpα1 and Hpα2), resulting in three main phenotypes: Hp 1-1, 2-1 and 2-2. The Hpα1 allele can be subdivided into two groups, a fast (Hpα1F) and slow (Hpα1S) form, that differ slightly in amino acid composition, resulting in different electrophoretic mobility's[75]. The Hpα2 allele contains a unique nonhomologous crossing-over in a part of the Hpα1F and Hpα1S alleles[76] and can only be found in man. Haptoglobin proteins form multimers due to disulfide bridges between two α-chains and the size of the multimers is determined by the α-chain phenotype[77] (figure 4). The Hpα1-chain is monovalent and can only form a disulfide bridge with another α-chain resulting in a haptoglobin tetramer $(\alpha 1\beta)_2$. In contrast, the Hpα2-chain is divalent and can form two disulfide bridges. Thus, Hpα2-chains can bind to either Hpα1- or Hpα2-chains to form large multimers $((\alpha_1\beta)_2\text{-}(\alpha_2\beta)_n$ in Hp2-1 phenotypes and $(\alpha_2\beta)_n$ in Hp2-2 phenotypes).

Besides the polymorphism, an additional haptoglobin gene has been isolated which shares a high degree of nucleotide sequence homology with

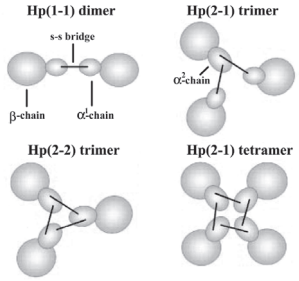

Figure 4. Schematic representation of the structural differences of haptoglobin multimers as determined by haptoglobin phenotype

Hpα1. This haptoglobin-related gene (Hpr) is thought to be evolved by gene duplication and subsequent divergence[78] and plays an important role in killing trypanosomes[79].

Haptoglobin in cardiovascular disease.
Haptoglobin phenotype was generally determined as a genetic fingerprint for the use in forensic medicine. More recently, the possession of a certain haptoglobin phenotype has been associated with a variety of common diseases, suggesting that there are functional differences between the haptoglobin phenotypes[80-81].
Cardiovascular disease is one of these diseases in which haptoglobin phenotype plays a role. There is a weak association between Hp2-1 phenotype and abdominal aortic aneurysm susceptibility[74, 82-83] which is thought to depend on increased elastase activity. Atherosclerosis and restenosis are, in contrast, associated with the Hp2-2 phenotype. Patients with this phenotype are more likely to develop restenosis after balloon angioplasty[84], stenting[85] or coronary surgery[86]. Moreover, there appears to be a gradient in the risk, with Hp2-1 individuals having an intermediate risk. Oxidative stress has been implicated in the initiation and pathogenesis of atherosclerosis[87] and might explain the prevalence of Hp2-2 phenotype in atherosclerosis as Hp1-1 appears to have better antioxidative capacity compared to Hp2-2[88-89]. Thus, specific haptoglobin phenotypes may offer protection against the development or progression of cardiovascular diseases.

Outline of the thesis:

The aim of this thesis is to gain more insight in the arterial expression and function of haptoglobin during arterial restructuring. In chapter 2, subtraction PCR identifies arterial haptoglobin expression after sustained flow changes and the role of haptoglobin in cell migration and arterial restructuring is inves-tigated. In chapter 3, the regulation of arterial haptoglobin expression after sustained flow changes is investigated. Chapter 4 describes the local expression of haptoglobin in arthritic and oncological tissues, two pathological processes characterized by extensive tissue remodeling and cell migration. Chapter 5 describes the existence of unique arterial haptoglobin β-chains, implying functional differences between arterial and liver haptoglobin. In chapter 6, the association between serum haptoglobin glycosylation patterns and atherosclerotic disease is investigated.

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I





The acute phase protein haptoglobin is a cell migration factor involved in arterial restructuring

Chapter II

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The acute phase protein haptoglobin is a cell migration factor involved in arterial restructuring.

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Collagen turnover and cell migration are fundamental aspects of arterial restructuring. To identify mRNAs involved in blood flow-induced arterial restructuring, we performed subtraction polymerase chain reaction and found expression of haptoglobin mRNA in arterial fibroblasts of rabbit arteries. Haptoglobin is highly expressed in liver, but it's arterial expression and function are unknown. In vitro studies revealed that stimulation of haptoglobin expression by lipopolysaccharides in mice fibroblasts stimulated migration of wild type fibroblasts, but had no effect on migration of haptoglobin knockout fibroblasts. In vivo studies showed that flow-induced arterial restructuring was delayed in haptoglobin knockout mice. This new function of haptoglobin might be explained by facilitating cell migration through accumulation of a temporary gelatin matrix because cell culture showed that haptoglobin is involved in the breakdown of gelatin. We conclude that haptoglobin is highly expressed in arterial tissue and is involved in arterial restructuring. This new haptoglobin function may also apply to other functional and pathological restructuring processes such as angiogenesis, tissue repair and tumor cell invasion.

Introduction

Proteolytic degradation and synthesis of the extracellular collagen matrix and cell migration are critical steps during tissue remodeling in normal and pathological processes, including wound healing, inflammation, tumor invasion and metastasis[1-2]. Tissue remodeling in arteries may induce a structural change in total arterial circumference and is a major determinant of luminal narrowing after balloon angioplasty[3-5], in atherosclerosis[6-7], and during sustained changes in blood flow[8]. Strength, stiffness, size and shape of the arterial wall are mainly determined by fibrillar collagen (type I and III) which is predominantly located in the adventitia[9]. Collagen breakdown by matrix metalloproteinases (MMPs) is essential for arterial restructuring to occur. Administration of MMP inhibitors can prevent arterial enlargement induced by a flow increase[10] as well as arterial shrinkage after balloon angioplasty[11-12].

Migration of adventitial fibroblasts and medial smooth muscle cells (SMC) is also a fundamental aspect of arterial restructuring. Translocation of cells from the adventitia or media to the intima is considered to be critical to the development of atherosclerosis[13] and restenosis after injury[14]. Locomotion of SMCs requires newly synthesized collagen, implicating a mechanism whereby integrin clustering and linkage to the cytoskeleton are required for effective cell movement[15]. Although collagen turnover and cell migration are regarded as essential processes of arterial restructuring, their components and regulatory mechanisms are still unclear.

The aim of this study was to identify components of collagen turnover and cell migration during flow-induced arterial restructuring. By using subtraction polymerase chain reaction (PCR) in rabbits, we found increased haptoglobin mRNA levels in adventitial fibroblasts. In vitro and in vivo assays showed that haptoglobin promoted cell migration and that in haptoglobin knockout mice, arterial restructuring was delayed compared to wild type mice. Cell culture showed that haptoglobin is involved in the degradation

of gelatins, the intermediate breakdown products of collagen. Taken together, this study shows that haptoglobin is expressed in arteries and is involved in arterial restructuring, probably through the formation of a temporary gelatin-based matrix that enhances cell migration.

Materials and Methods

Animals

Rabbits: Surgical interventions to increase or decrease flow were performed in the right carotid and femoral arteries of each New Zealand White rabbit (Broekman Charles River, 3-3.5 kg). The untreated left femoral arteries served as control. Rabbits were anesthetized by intramuscular injection of methadon (0.15 ml) and Vetranquil (0.15 ml) followed by intravenous injection of etomidate (1 mg/kg) and ventilation with N_2O:O_2 (1:1) and 0.6% Halothane. An incision was made in the neck to expose the right common carotid artery and jugular vein. For the femoral artery and vein, an incision was made in the upper leg.

To increase the flow by about a factor 10, a side-to-side anastomosis was made between the artery and the vein resulting in arterial enlargement[16]. To reduce flow by about half, a partial ligation was made by placing a constricting suture around the artery until flow was reduced to at least 60% of the initial value, resulting in arterial shrinkage[16]. Rabbits were killed 1, 2 and 7 days after the operation. At operation and termination, blood flow was measured by using a transit time flow probe (Transonic System Inc.). Arterial segments to be analyzed were dissected from the artery not closer than 1.5 cm from the operation area.

Mice: The haptoglobin knockout mice had been back-crossed into the wild type mice BALB/c background for at least 12 generations at the time of the experiment. Female wild type BALB/c mice and BALB/c mice with a haptoglobin null mutation[17] were anesthetized by using 0.05 ml/10 g body weight of a cocktail (1 part Hypnorm, 1 part Midazolam, 2 parts distilled water). The right common carotid artery was ligated as described by Kumar and Lindner[18]. Animals were killed at 5, 8 and 20 days after ligation. Before the arteries were harvested, they were perfused via the left ventricle for 3 min with phosphate buffered saline (PBS) plus 10^{-4} M sodium nitroprusside followed by a 3 min perfusion with 4% paraformaldehyde in PBS plus 10^{-4} M sodium nitroprusside at 99 ml/h. After the harvest, the arteries were fixed in 4% paraformaldehyde in PBS for several days before being embedded in paraffin. Eight or more serial sections (6 µm), spanning most of the vessel segment, were cut and stained with elastin von Gieson and hematoxilin-eosin for morphometry. All sections were analyzed for morphometry using the SIS analysis software package.

The investigation conformed to 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 Faculty of Medicine, Utrecht University, and University of Singapore.

Extraction of RNA and protein

After collection of the rabbit artery, a small part was dissected and fixed for 2 h in 4% paraformaldehyde at room temperature (RT), followed by an overnight (%) incubation at 4 °C in 15% sucrose in PBS. Samples were embedded in Tissue Tec (Sakura Fineteck) and stored at –80 °C until use for in situ hybridization or immunohistochemistry. The remaining artery was frozen and ground with a pestle and mortar in liquid nitrogen until a fine powder was obtained and was used for Tripure (Roche) isolation of RNA and protein. After collection of the mouse carotid artery, the complete artery was used for RNA and protein extraction. Extraction and purification of total RNA and protein was performed according to manufacturer's protocol.

Quantitative PCR

PCR amplification was performed for the mouse carotid arteries and rabbit carotid arteries and liver with the I-cycler iGTM Real Time PCR (Bio-rad). Each reaction contained 14 µl of cDNA, 200µM dNTP, 1x reaction buffer (InVitrogen) containing 1:80,000 Cybergreen (Bio-rad), 2.5 U of Taq DNA polymerase (InVitrogen) and each primer at 1 µM. Quantities were determined by comparison with known quantities of the cloned PCR products representing the target mRNAs. Data were corrected for the amount of 18S mRNA that was used as an internal standard.

The following oligonucleotides were used as primers: mouse haptoglobin (forward 5'-AAAAACCTCTTCCTGAACCAC-3', reverse 5'-AACGAC CTTCTCAATCTCCAC-3'), mouse 18s (forward 5'-TCAACACGGGAA ACCTCAC-3', reverse 5'-ACCAGACAAATCGCTCCAC-3'), rabbit haptoglobin (forward 5'-GAAGCAGTGGGTGAACAAGG-3', reverse 5'-TGACAAGATTGTGG CGGGAG-3'), rabbit 18s (forward primer 5'-TCAACACGGGAAACCTCAC-3', reverse primer 5'-ACAAATCGCTC CAGCAAC-3').

Subtraction PCR, library screening and sequence analysis

Total RNA, isolated from one femoral artery after flow reduction and from its contralateral artery, was converted into cDNA and amplified using the SMART PCR cDNA Synthesis kit (Clontech). Samples were prepared for the PCR-Select cDNA subtraction kit according to manufacturer's protocol. Subtracted cDNA was then ligated in pGEM-T-easy (Promega) and transformed in Xl1blue by using supercompetent cells (Stratagene). White colonies were picked and individually grown %. Double-stranded plasmid DNA was isolated (Qiagen) and, after 1:100 dilution in water, spotted on four nylon membranes (Hybond-N, Amersham), resulting in four identical membranes.

After baking proceeded for 2 h at 80 °C, each blot was hybridized with a different cDNA probe: unsubtracted cDNA of the flow reduced artery, unsubtracted cDNA of the control artery, subtracted cDNA of flow reduction minus control and subtracted cDNA of control minus flow reduced artery. Clones were selected for sequence analysis when they showed a large difference between the two subtracted probes and no or a low signal for the unsubtracted probes.

Sequence analysis was performed on both strands using the T7 Sequenase 2.0 sequencing kit (Amersham).

Northern and Western blotting

For Northern blotting, hybridization occurred at 65 °C in Easy-hyb hybridization solution (Stratagene) for 1 h in a hybridization oven. The membrane was washed in 0.1 x standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 65 °C for 10 min.

For Western blotting, the amount of protein was determined by using the Biorad DC Protein assay. Protein samples (8 or 3 µg/lane for the mice carotid arteries) were separated on a SDS polyacrylamide gel (8%). After electrophoresis, the gel was blotted to Hybond-C (Amersham) in blotting buffer (3.03 g /L Tris 14.4 g/L Glycine, 20% methanol). After the blotting, the gel was blocked % in PBS plus 0.1% Tween 20 (PBST) and 5% non-fat dry milk (Protifar, Nutricia). Incubation with the first antibody (goat-α-human collagen 1:1000, SBA or goat-α-human haptoglobin 1:4000, ICN) occurred in PBST plus 5% Protifar for 1 h at RT followed by washing (three times for 5 min each time) with PBST. Incubation was continued with rabbit-α-goat biotin (1:1000, DAKO) for 1 h at RT in PBST plus 5% Protifar followed by washing (three times) with PBST. After this, incubation with streptavidin-peroxidase (1:2000, Southern Biotechnology) occurred at RT for 1 h in PBST plus 5% Protifar. The blot was washed two times with PBST. Bands were visualized by using the enhanced chemiluminescence kit (Amersham).

In situ hybridization

Linearized R-1045 in PGEM T-easy was used as a template to obtain digoxigenin (Dig, Roche) labeled RNA probes. Arterial segments were cut into 8 µm sections and were transferred to Superfrost plus slides (Menzel Glazer) and stored at –80 °C until use.

After the sections were defrosted, they were treated with 0.2 M HCl for 20 min at RT, washed three times with PBS for 5 min and treated with proteinase K (Roche, 10 µg/ml) for 10 min at 37 °C in PBS. The sections were then washed with PBS and fixed with 4% paraformaldehyde for 5 min at RT and treated twice for 5 min with acetic anhydride in triethylacetate (TEA, 185 µl acetyl anhydride in 0.1 M TEA). Sections were subsequently washed twice in 2 x SSC for 5 min at RT, followed by 5 min in 2 x SSC/50% formamide at 37 °C.

For prehybridization, 100 µl of hybridization mix (50 % formamide, 1mg/ml tRNA, 1 x Denhardts, 10% dextrane sulfate, 4 x SSC) was added to the slide and the slide was incubated 1 h at 46 °C. A Dig (1 µl)-labeled RNA probe was added, and hybridization continued % at 46 °C.

After hybridization, sections were washed in 0.1 x SSC at 45 °C for 15 min, followed by RNAse treatment (40 µg/ml RNAse A, 1 mM EDTA pH 8, 2 x SSC) for 15 min at RT, and then sections were washed again in 0.1 x SSC at 45 °C for 15 min. Before detection, sections were rinsed

with 2 x SSC and 100 mM Tris pH 7.4 in 150 mM NaCl at RT. Detection was performed according to manufacturer's protocol.

Immunohistochemistry
Immunohistochemistry was performed with sections treated according the aforementioned method and used after in situ hybridization. Sections were fixed for 10 minutes in acetone containing 0.03 % H_2O_2 to block endogenous peroxidase. Next, sections were incubated with 10% normal goat serum for 30 minutes (RT) and, in addition, were incubated with 5 µg/ml mouse-α-vimentin monoclonal antibody (Sigma) $^{o}/_{n}$ at 4°C in PBS/0.1% bovine serum albumin (BSA). After $^{o}/_{n}$ incubation, sections were rinsed in PBS (three times for 5 min) and were incubated with 1 µg/ml goat-α-mouse peroxidase polyclonal antibody (DAKOpatts) in PBS/1% BSA containing 1% normal rabbit serum (1 h, RT). Sections were next rinsed in PBS (three times for 5 min). To visualize the peroxidase, sections were treated for 10 min with a sodium acetate buffer containing 0.4 mg/ml 3-amino-9-ethylcarbazole substrate.

Mouse primary fibroblasts
Mouse primary fibroblasts were prepared as previously described[19]. Mouse primary fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) (InVitrogen) with 1x MEM nonessential amino acids (InVitrogen), 3.7 µl/500 ml β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin and 20% fetal bovine serum (FBS) (InVitrogen). Genotyping was done as described earlier[17].
Migration was studied by using uncoated transwell culture chambers (Costar 3422) with approximately 20,000 cells per chamber. Incubation of cells with 10 ng/ml lipopolysaccharide (LPS) started 24 h before the migration assay and was continued during the assay. Control cells were incubated in the same media without LPS. Migration was continued for 20 h without chemoattractant according to manufacturer's protocol. The cells were counted as migrated cells when they migrated and attached to the bottom well culture dish.

Primary human adventitial fibroblasts
A small part of the thoracic aorta was dissected from human donor and recipient hearts during heart transplantation. The adventitial layer was dissected from the aorta and rinsed several times in PBS, and cells were isolated by using collagenase. Cells were cultured in DMEM (InVitrogen) supplemented with L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% FBS (InVitrogen) and were used throughout passages two to four.

Cell culture
Human arterial SMCs (American Type Culture Collection: CRL 1999, passage 22-26) were used because they produce only small amounts of haptoglobin (data not shown). Cells were grown in Ham's F12 medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10 mM HEPES, 10 mM TES, 50 µg/ml ascorbic acid, 10 µg/ml insulin, 10 µg/ml transferrin, 10 ng/ml sodium selenite, 30 µg/ml endothelial cell growth supplement and 10% FBS. During experiments, 1% FBS or mouse serum was used. After this, the SMC culture medium with or without haptoglobin was harvested and used for Western blotting with an antibody directed against human collagen I and III (Sigma).

MMP-2 activity, gelatinase assay, and zymography
MMP-2 activity assay: Different concentrations of haptoglobin (Sigma) or BSA (Bio-rad) were added to 1 ng of activated MMP-2 or MMP-9 by using the MMP-2 or MMP-9 activity assay (Amersham). The assay was performed according to manufacturer's protocol.
Gelatinase assay: Polyacrylamide gels (8%) containing 1 mg/ml gelatin (Sigma) and Brij solution (0.05 M Tris-HCl pH 7.4, 0.01 M $CaCl_2$, 0.05% Brij 35 (Sigma)) were used, in which F12 medium or 10^{-6} M collagen I (Biogenesis) or 10^{-6} M haptoglobin (Sigma) or marimastat (100 ng/ml, British Biotech) were added to the gels. Samples of 1 µl of purified MMP-2 or MMP-9 (Biogenesis) were spotted on the gel in different concentrations and the gel was incubated at 37 °C for 8 h. The gel was then stained with Coomassie brilliant blue (25% methanol, 15% acetic acid, 0.1% Coomassie brilliant blue) for 1 h at RT, followed by destaining (25% methanol, 15% acetic acid) for approximately 30 min. MMP-activity was quantified by the amount of gelatin degradation (optical density/mm^2) using the Gel Doc 1000 system (Bio-rad). Zymography: Zymography was performed as described earlier[16] with 5 µl of medium from heterozygous or knockout murine embryonic fibroblasts.

Statistics
Data are presented as mean ± standard deviation. Student's *t* test plus a Bonferroni correction was used for differences between mean values. $P<0.05$ was considered as statistically significant.

Results

Subtraction PCR with rabbit mRNA isolated from the partially ligated femoral artery (blood flow decrease) and its contralateral artery (unchanged blood flow) revealed 30 clones encoding 20 different mRNAs. Clone R1045 with a sequence of 647 bp was identified as a known protein. The open reading frame coded for a partial protein of 197 amino acids (EMBL: AJ250102) with a high identity to haptoglobin reported previously for the monkey, human, mouse and rat haptoglobin (85%, 84%, 82% and 80%, respectively).

Localization of haptoglobin in rabbit arterial wall

Nonradioactive in situ hybridization showed clear staining of a large number of cells in the adventitial layer of the carotid artery at 1 day after blood flow increase (figure 1A), close to the external elastic lamina (figure 1C). No staining was found in the sense control (figure 1B) and in naïve arteries (not shown). Interestingly, a few cells containing haptoglobin mRNA were also found in the medial layer (figure 1D), which suggests migration of cells from the adventitia to the media.
To identify the haptoglobin-producing cell type, we performed double staining of haptoglobin mRNA and vimentin to discriminate fibroblasts from macrophages. Figure 1E demonstrates the black staining of haptoglobin mRNA, whereas figure 1F shows, at another depth of field, the more prominent red vimentin staining.

Haptoglobin expression in rabbit tissue, rabbit arteries after blood flow changes, and human cells

To determine whether the same haptoglobin mRNA was expressed in artery and liver, a tissue blot (figure 2A) was used to show the bands of the same size in carotid artery and liver. Quantitative real-time PCR was used to determine relative amounts of haptoglobin mRNA in arteries and liver. This assay showed that 200 ng of total

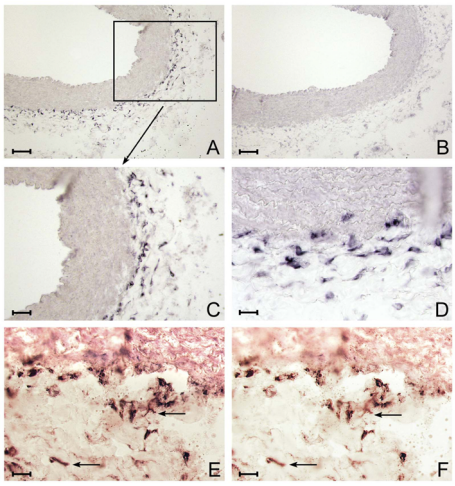

Figure 1. Localization of haptoglobin mRNA in rabbit adventitial fibroblasts.
A-B) In situ hybridization of rabbit carotid artery sections using an antisense cRNA probe against rabbit haptoglobin mRNA (A) and a sense control cRNA probe (B). Scale bar=125 µm. **C)** Detail of the indicated area in A using an antisense cRNA probe against haptoglobin mRNA. Scale bar=50 µm. **D)** Detail of haptoglobin mRNA-producing cells in adventitia (bottom layer) and media (top layer), shown by an antisense cRNA probe. Scale bar=12.5 µm. **E-F)** In situ hybridization and immunohistochemical double staining of rabbit carotid artery sections by using an antisense cRNA probe against haptoglobin mRNA and an antibody directed against vimentin. **E)** Black staining of haptoglobin mRNA. **F)** At another depth of field, the more prominent red vimentin staining. Arrows indicate cells stained for haptoglobin mRNA (E) and vimentin (F) in the adventitial layer. Scale bar=12.5 µm.

RNA of a control carotid artery contained 2±4% (n=4) of liver haptoglobin mRNA (100%), whereas 200 ng of total RNA of a carotid artery 1 day after ligation contained 38±46% (n=4) of liver haptoglobin mRNA.
After a sustained blood flow increase (an increase of about 10 times) as well as after a sustained blood flow decrease (a decrease of about 0.5 times), haptoglobin mRNA was found in the rabbit arterial wall. Both increased and decreased blood flows in the right carotid artery were accompanied by increased blood flow in the left carotid artery. Without an operation on the left carotid artery, blood flow increased to compensate for the diminished blood flow to the brain on the right side[20]. No blood flow changes occurred in the untreated left femoral artery, which served as a control.
One day after the blood flow increase, haptoglobin mRNA expression was highest (figure 2B, C). In the right femoral artery, haptoglobin mRNA levels increased more then 100 times ($p<0.0001$) compared with it's contralateral control artery (figure 2E, G). The haptoglobin mRNA levels in the right and left carotid artery were also increased more then 100 times ($p=0.05$ and $p=0.03$, respectively) compared with the control left femoral artery (figure 2E, G). Haptoglobin mRNA levels, determined by quantitative PCR, were the same in the control left femoral artery and a femoral artery of an unmanipulated animal (results not shown).
To determine haptoglobin protein levels after a sustained blood flow increase, the same arteries were also used for Western blot analysis with an antibody directed against human haptoglobin (figure 2F, H). This assay revealed an approximate sevenfold increase in haptoglobin protein in the operated right femoral artery compared with the contralateral control artery ($p=0.09$). Increases in haptoglobin protein levels of about fivefold were also observed in the right and left carotid arteries compared with the control femoral artery ($p=0.03$ and $p=0.12$, respectively). Haptoglobin was also present in human primary adventitial fibroblasts (figure 2D) and was secreted into the medium.

Haptoglobin and cell migration

As cell migration is an essential process in arterial restructuring, we used mice embryonic fibroblasts to investigate a potential role of haptoglobin in cell migration. The absence of haptoglobin in haptoglobin knockout embryonic fibroblasts (figure 3A) resulted in slower migration ($p=0.03$) of the fibroblasts as compared with wild type and heterozygous embryonic fibroblasts (figure 3B). Furthermore, incubation of embryonic fibroblasts with LPS induced an increase in haptoglobin mRNA expression in wild type and heterozygous fibro-

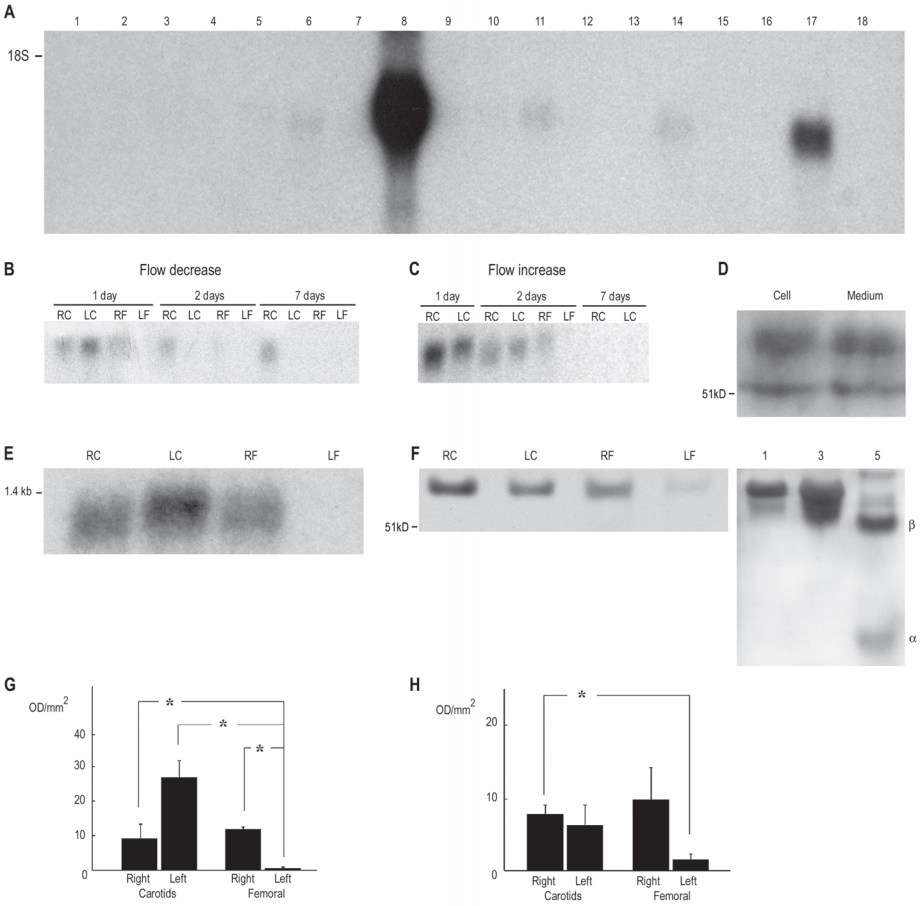

Figure 2. Tissue expression of haptoglobin in rabbit and human cells
A) Tissue expression of haptoglobin mRNA in different rabbit tissues. Northern blot analysis of 20 µg of total RNA per lane. Lanes: 1 aorta, 2 adrenal gland, 3 cerebellum, 4 frontal cortex, 5 heart, 6 hypothalamus, 7 mesenteric artery, 8 liver, 9 lung, 10 spleen, 11 neural retina, 12 kidney, 13 ovary, 14 renal artery and 15 thyroid gland; 10 µg of total RNA of buffy coat fraction in lane 16, carotid artery after 2 days of sustained flow changes in lane 17 and erythrocyte fraction in lane 18. The position of the 18S band is indicated. In right carotid (RC) and right femoral (RF) arteries, blood flow changes were created surgically. In the left carotid (LC) artery, blood flow increased to compensate for the diminished blood flow to the brain on the right side (20). No blood flow changes occurred in the untreated left femoral (LF) artery which served as a control. Typical results of Northern blot analysis (10 µg/lane) are presented in B, C and E and of Western blot analysis (8 µg/lane) in F. **B)** Haptoglobin mRNA expression in rabbit arteries after 1, 2 and 7 days of sustained blood flow decrease obtained by partial ligation. **C)** Haptoglobin mRNA expression in rabbit arteries after 1, 2 and 7 days of sustained blood flow increase obtained by arterio-venous shunting. **D)** Western blot of cell lysate (8 µg/lane) and medium (10 µg/lane)from human primary adventitial fibroblasts stimulated for 24 h with 10 ng/ml of LPS. **E)** Haptoglobin mRNA expression in rabbit arteries 1 day after blood flow increase. **F)** Left panel: haptoglobin protein expresion in rabbit arteries 1 day after blood flow increase. Right panel: denaturation of the right carotid haptoglobin complex after 1 min at 100 °C (1), 3 min at 100 °C (3) and 5 min at 100 °C (5). Haptoglobin α– and β–subunits are indicated. **G)** Relative haptoglobin mRNA levels in arteries of four rabbits determined by using Northern blot analysis (10 µg/lane). OD, optical density (arbitary units). **H)** Relative haptoglobin protein levels in arteries of four rabbits determined by using Western blot analysis (8 µg/lane). Protein was extracted from the same arteries as used for G. The asterisk indicates statistical significance ($P<0.05$).

blasts (figure 3A). As expected, no expression was found in haptoglobin knockout cells. Concomitant with the increase in haptoglobin production, LPS treatment also increased the number of migrating wild type and heterozygous cells 1.5-2 times (p=0.02). The haptoglobin knockout cells, in contrast, showed no increase in the number of migrating cells (figure 3B).
Because haptoglobin is secreted, we reasoned that medium from wild type fibroblasts might rescue migration of the knockout cells. A migration assay was performed with haptoglobin knockout cells and wild type cells (figure 3C). Again, incubation with LPS stimulated migration of only the wild type cells, but the number of migrating knockout cells increased (p=0.03) after incubation in medium from wild type cells.

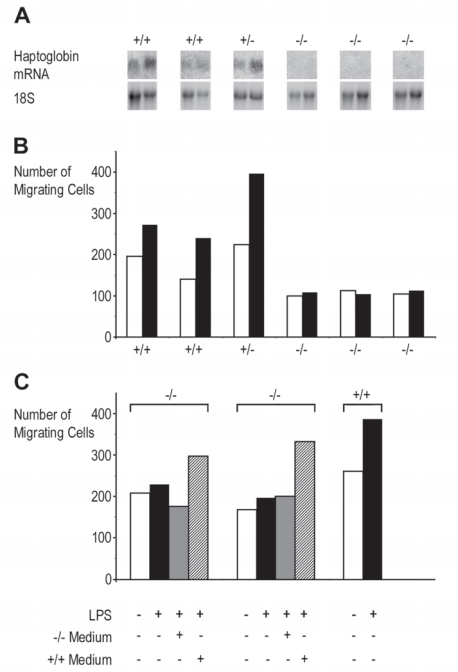

Figure 3. Haptoglobin and cell migration
A-B) Haptoglobin expression and cell migration of murine primary fibroblasts cell lines. **A)** Northern blot analysis of RNA (approximately 10 µg /lane) from six mice embryonic primary fibroblasts cell lines with different haptoglobin genotypes (+/+ = wild type, +/- = heterozygous, -/- = knock out). Top panels: Haptoglobin mRNA expression in primary fibroblasts of six different embryos without LPS (left lane) or with 10 ng/ml LPS (right lane). Bottom panels: 18S ribosomal bands of lanes in top panels. **B)** Migration assay with primary fibroblasts of the same embryo's as used in A. Approximately $2x10^4$ cells were used per chamber with 10 ng/ml LPS (black bars) or without LPS (white bars), without chemoattractant. **C)** Cell migration of haptoglobin -/- fibroblasts. Haptoglobin -/- fibroblasts from two embryos were incubated during the migration assay without LPS (white bars), with 10 ng/ml LPS (black bars), with 10 ng/ml LPS and medium from haptoglobin -/- fibroblasts incubated 16 h with 10 ng/ml LPS (grey bars) or with 10 ng/ml LPS and medium from haptoglobin +/+ fibroblasts incubated 16h with 10 ng/ml LPS (striped bars). Haptoglobin +/+ fibroblasts were used as a positive control.

Unilateral common carotid ligation in wild type and haptoglobin knockout mice

A murine carotid ligation model[18] was used to investigate whether arterial restructuring was affected in haptoglobin knockout mice. Carotid arteries were obtained at 5, 8 and 20 days after cessation of blood flow.
At 5 days, morphometry showed no differences in intimal, medial and adventitial cross-sectional areas between wild type and haptoglobin knockout mice (figure 4A). Hematoxylin-eosin staining (figure 4B), however, showed that morphological changes occurred in all three layers in both groups as described earlier[18]. In short, intimal lesions were detached from the underlying internal elastic lamina (IEL), thereby forming spaces filled with red blood cells, leukocytes and macrophages. In the media, the SMC structure disappeared and inflammatory cell infiltration was observed. The latter occurred also in the adventitia. At 8 days, morphometry showed that the intimal and adventitial areas of the carotid arteries of the knockout mice were enlarged (figure 4A) compared with those of the wild type mice. Arteries from the haptoglobin knockout mice still had a detached intimal area, abnormal SMC structure in the media and infiltration of inflammatory cells in all three arterial layers. The wild type mice, in contrast, showed almost no intima hyperplasia and medial SMC morphology appeared normal[18]. Throughout the arterial wall, no inflammatory cells were found.
At 20 days, morphometry and hematoxylin-eosin staining (figure 4A, B) showed no differences between the carotid arteries of the haptoglobin knockout mice and the wild type mice. The artery contralateral to the ligated artery showed no neointima formation and no morphometric differences between wild type and haptoglobin knockout mouse (results not shown). Elastin von Gieson staining showed that the internal and

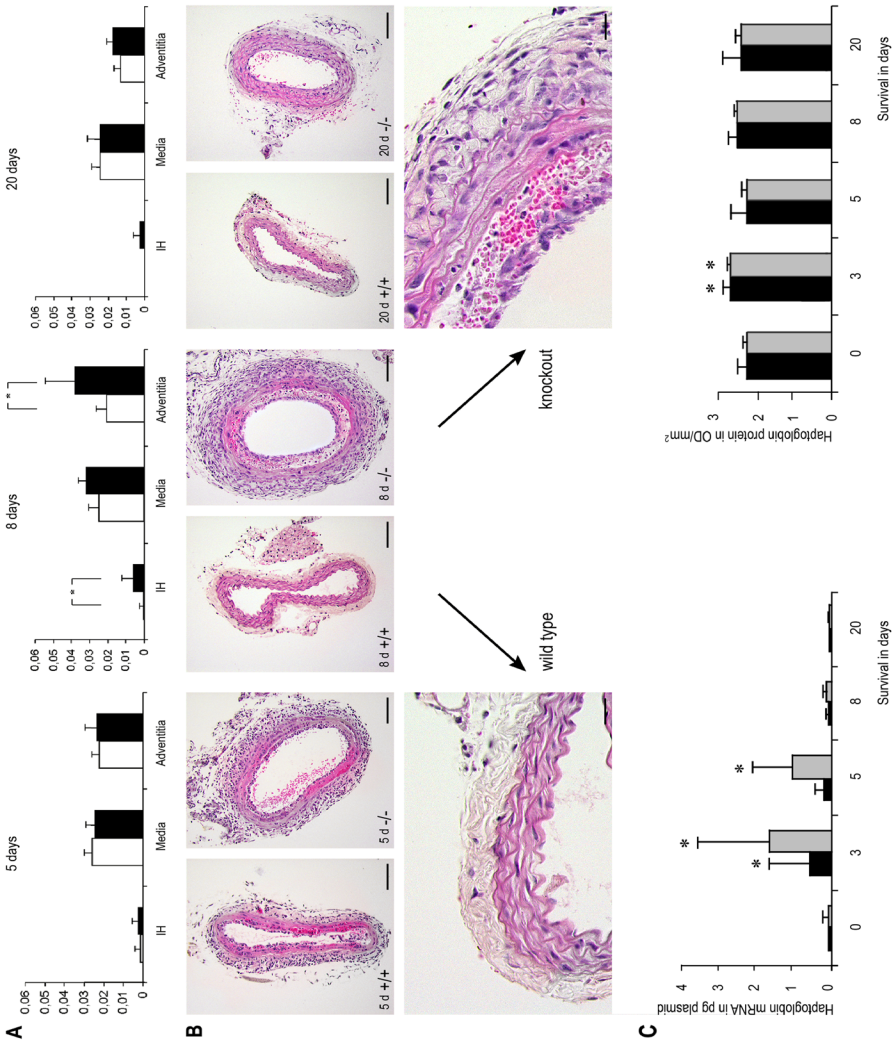

Figure 4. Arterial restructuring in a murine blood flow cessation model using wild type and haptoglobin knockout mice

A) Cross-sectional wall layer areas (mm^2) 5, 8 and 20 days after unilateral ligation of the common carotid artery in wild type female BALB/c mice (white bars) and female BALB/c mice with a haptoglobin null mutation (black bars). Intimal hyperplasia (IH), medial area (Media) and adventitial area (Adventitia) were measured and averaged using digital analysis of at least eight sections per artery. N=5-7 mice per group. Data represent mean±standard deviation. The asterisk indicates statistical significance ($p<0.05$). **B)** Hematoxylin-eosin staining of representative sections of the common carotid artery after unilateral ligation. The top six panels represent carotid arteries 5, 8 and 20 days after ligation. Scale bar=50 µm. The two bottom panels are enlargements of the carotid artery of a haptoglobin knockout mouse (right) or a wild type mouse (left) 8 days after ligation. Scale bar=12.5 µm. **C)** Haptoglobin mRNA and protein expression in left (black bars) and right (grey bars) carotid arteries from wild type female BALB/c mice at 0, 3, 5, 8 and 20 days after right carotid ligation. Haptoglobin mRNA is presented as the amount of plasmid containing the haptoglobin PCR product to which it correlates in the dilution series of this plasmid used in the quantitative real-time PCR. N=9-10 arteries/time point. The asterisk indicates statistical significance ($p<0.05$) compared with 0 days.

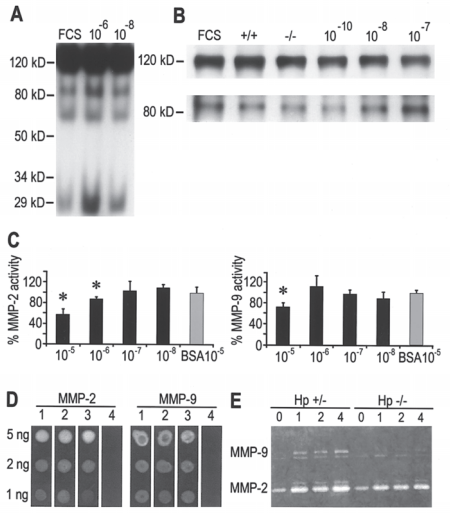

Figure 5. Haptoglobin and in vitro collagen breakdown
A-B) Western blot analysis of human arterial SMC (ATCC: CRL 1999) culture medium after 24 h of incubation with various concentrations of human haptoglobin protein (Biogenesis) using an antibody directed against human collagen. **A)** Western blot analysis of cell culture medium containing 1% FCS (FCS), 1% FCS plus 10^{-6} M haptoglobin (10^{-6}) or 1% FCS plus 10^{-8} M haptoglobin (10^{-8}). **B)** Western blot analysis of cell culture medium containing 1% FCS (FCS), 1% serum from wild type C57BL/6J mice (+/+), 1% mouse serum from haptoglobin knockout C57BL/6J mice (-/-) or 1% serum from haptoglobin knockout mice plus 10^{-10} M haptoglobin (10^{-10}), 10^{-8} M haptoglobin (10^{-8}) or 10^{-7}M haptoglobin (10^{-7}). Top panel: short exposure of the procollagen band (120 kDa) in the different media. Bottom panel: longer exposure of the smaller collagen product (80 kDa) using the same blot as in the top panel. **C)** Relative inhibition (in %) of MMP-2 (left panel) and MMP-9 (right panel) activity with 10^{-5}, 10^{-6}, 10^{-7} or 10^{-8} M human haptoglobin compared with 10^{-5} M BSA. Asterisk indicates statistical significance ($P<0.05$). **D)** Example of gelatinase activity after spotting 1 µl of Brij solution containing 1, 2 or 5 ng of MMP-2 or MMP-9 protein on acrylamide gels. Gel 1 contained 8% acrylamide, 1 mg/ml gelatin, and F12 culture medium. Gel 2: same as gel 1 plus 10^{-6} M collagen in F12 medium. Gel 3: same as gel 1 plus 10^{-6} M haptoglobin in F12 medium. Gel 4: same as gel 1 plus 100 ng/ml marimastat, the MMP inhibitor, in F12 medium. **E)** Zymographic determination of MMP-2 and MMP-9 activity in medium (containing 0.1% FCS) from culture of mouse embryonic fibroblasts heterozygous (+/-) or deficient (-/-) for haptoglobin. Medium was incubated with the fibroblasts for 0, 1, 2 and 4 h (n=4).

external elastic laminae were intact in all arteries. Haptoglobin mRNA and protein expression in carotid arteries from wild type female BALB/c mice after right carotid ligation (figure 4C) showed an increase at 3 days in the right and left carotid arteries. In the ligated right carotid artery, haptoglobin mRNA was still increased at 5 days after ligation. In this right carotid artery, gelatinase activity was also increased at 3 days (approximately 20 times, p=0.001).

Haptoglobin and collagen breakdown

Having demonstrated that haptoglobin is involved in cell migration and arterial restructuring, we studied whether haptoglobin could effect collagen breakdown because this is an important feature of both processes. The human arterial SMC secretes procollagen, MMPs and only a little haptoglobin compared with fibroblasts (results not shown). Newly synthesized procollagen is proteolytically cleaved to mature collagen and is built into the collagen fiber or degraded by collagenases into two gelatin breakdown products, which are subsequently degraded by gelatinases.

Human SMCs were cultured and the medium was changed to a medium containing only 1% FBS with different concentrations of human haptoglobin. Western blotting revealed the presence of procollagen proteins (130 kDa) and two main smaller collagen proteins (80 kDa and 30 kDa) (figure 5A). When 10^{-6} or 10^{-8} M haptoglobin was added to the culture medium, procollagen levels did not change, but the levels of the two smaller collagen products increased. In addition, we used serum from the haptoglobin knockout mouse as a negative control and added haptoglobin in increasing concentrations (figure 5B), which produced an increase of the 80-kDa band when the haptoglobin concentration increased.

On the basis of the primary collagenase cleavage site, the molecular size of the collagen products was almost equivalent to the calculated size of the collagenase degradation (gelatin) products of procollagen α1(I) and α2(I) (79.1 kDa and 26.4 kDa; 76.5 kDa and 23.6 kDa, respectively[21]). To assess whether the increase in intermediate collagen breakdown products was due to inhibition of gelatinase activity, we studied the in vitro effect of haptoglobin on the gelatinases MMP-2 and MMP-9 in an activity assay (figure 5C) and a gelatinase assay (figure 5D). The MMP-2 and MMP–9 activity assays revealed an inhibition in MMP-2 activity of about 50% (p=0.004) for 10^{-5} M haptoglobin and about 20% (p=0.04) for 10^{-6} M haptoglobin. MMP-9 activity decreased by about 30% (p=0.02) when 10^{-5} M haptoglobin was added.

Gelatin degradation by MMP-2 or MMP–9 was studied in the presence of 10^{-6} M haptoglobin, 10^{-6} M collagen I or F12 medium. The MMP inhibitor marimastat was used as a negative

control. When haptoglobin was present, the breakdown of gelatin was inhibited to 70% and 40% of the collagen control when 1 ng and 2 ng of MMP-2 was added, respectively.
Zymography of medium from mouse embryonic fibroblasts heterozygous (+/-) or deficient (-/-) in haptoglobin (figure 5E) showed a stronger increase in activity of MMP-2 (1.3-1.8 times) and MMP-9 (3.4-13.8 times) when the medium was incubated for 1, 2 and 4 h with +/- cells compared with the -/- cells.

Discussion

Haptoglobin, an acute phase protein, is produced mainly in the liver where it is released into the plasma. It is generally considered to be important for rapid hepatic clearance of hemoglobin from plasma. Ahaptoglobinaemia, however, does not cause impaired hemoglobin clearance[22], a feature also seen in haptoglobin knockout mice. In contrast, the endocytosis of hemoglobin by hemoglobin scavenger receptor CD163 in macrophages can only occur via haptoglobin-hemoglobin complexes[23]. During hemolysis, however, haptoglobin knockout mice suffer greater renal tissue damage and fail to repair damaged renal tissue[17,24]. This observation points to a potential role for haptoglobin in tissue repair. Other studies on haptoglobin indicate that it may be involved in other extracellular matrix-related processes. Malignant ovarian tumors[25] and abdominal aortic aneurysms[26] are associated with elevated haptoglobin plasma concentrations. In vitro studies indicate a role for haptoglobin in bone resorption[27] and inflammation[28-29]. Moreover, haptoglobin stimulates angiogenesis both in vitro and in vivo[30] and a haptoglobin-like mRNA may be associated with localized angiogenesis in human endometrium[31].

Haptoglobin expression in the arterial wall

Expression of haptoglobin in arterial tissue has not been reported before. In our studies reported here, both a sustained increase and a decrease in blood flow induced haptoglobin mRNA and protein expression.
Altered shear stress and not wall tension appears to be the main trigger for haptoglobin expression, as no differences were found in mRNA levels in arterial segments proximal (increased wall tension) and distal (decreased wall tension) to the partial ligation (results not shown). Haptoglobin expression in the nonoperated left carotid artery, in which flow was increased because of collateral flow, demonstrated that the blood flow change and not the surgery initiated haptoglobin expression.
Haptoglobin mRNA expression was found in the adventitia. Because no SMCs are present in the adventitia, the colocalization with vimentin showed that haptoglobin was produced in adventitial fibroblasts and not macrophages.

Haptoglobin function

To investigate the role of haptoglobin in cell migration, we incubated mouse fibroblasts with LPS, which increased haptoglobin expression and stimulated cell migration of wild type but not of haptoglobin KO fibroblasts. Moreover, medium from wild type fibroblasts increased migration of knockout cells after LPS stimulation, which demonstrates that haptoglobin is involved in cell migration.
By using carotid ligation in haptoglobin knockout mice, cellular rearrangement and migration was delayed, resulting in delayed repair of the arterial wall and a prolonged inflammatory response, in accordance with the time course of carotid haptoglobin expression in wild type mice. Haptoglobin expression was the highest in the ligated right carotid artery, which showed a strong inflammatory response at 5 days, but the left carotid artery, which did not have an inflammatory response, also showed increased haptoglobin expression. Thus, haptoglobin, because of its involvement in cell migration and arterial repair, probably also has an anti-inflammatory function.
No morphological changes in the nonligated left carotid artery were found in wild type and haptoglobin knockout mice. This result does not exclude the possibility that haptoglobin is involved in structural arterial dilation but probably points to a backup mechanism for this important physiological process.
Intimal hyperplasia was minimal in wild type BALB/c mouse at 20 days. This finding may be due to an earlier time-point used here (20 days in

this study versus 28 days in the Lindner model) and another mouse strain (BALB/c versus FVB) with less intimal formation[32]. Gelatinase activity was increased when haptoglobin expression was highest, indicating that there is an association between haptoglobin expression and gelatinase activity in this mouse ligation model.

Appreciating the essential role of collagen in blood flow-induced arterial remodeling and cell migration, together with the association of haptoglobin and extracellular matrix-related processes, we hypothesized that haptoglobin is involved in collagen turnover. In vitro, arterial SMCs showed an increased production of the 80- and 30-kDa gelatin products when haptoglobin was added to the incubation medium. As no changes in procollagens were found, we presumed that haptoglobin inhibits gelatinases such as MMP-2 and MMP–9, which were the major gelatinases in the SMC culture medium (results not shown). An in vitro gelatinase assay and a MMP-2 and MMP-9 activity assay both confirmed this assumption by showing a decrease in MMP-2 and MMP –9 activity when haptoglobin was added. The increase in MMP-2 and MMP–9 activity in medium of heterozygous embryonic fibroblasts is probably due to a feedback loop. The increase in MMP-9 activity is higher than the increase in MMP-2 activity. This result is probably due to a more rapid response of MMP-9 than MMP-2 in the feedback loop and agrees with the early response of MMP-9 activity compared with MMP-2 activity in a murine carotis flow cessation model[33]. To get the same amount of gelatin breakdown, more MMPs are needed when haptoglobin is present. Because haptoglobin is separated from the MMPs in the zymogram, this will result in an increase in gelatinase activity. This result agrees with zymographic results with pig arteries treated with or without the broad-spectrum MMP inhibitor marimastat, showing an increase in MMP-2 and MMP-9 activity after MMP inhibition (unpublished data). However, a modified MMP activity assay showed that there was indeed less MMP activity in the same MMP-inhibited arteries[12]. The increase in MMP-2 and MMP–9 activity in medium of haptoglobin-producing embryonic fibroblasts confirms again the relationship between haptoglobin and gelatinase activity but also shows that regulation of gelatinase activity by haptoglobin is complicated and needs further investigation.

These results point to a new concept in which haptoglobin-induced accumulation of gelatin serves as a temporary matrix for cell migration, analogous to the temporary fibrin matrix in angiogenesis[34]. This hypothesis is in accordance with the finding that inhibition of collagenases and gelatinases, via either a recombinant tissue inhibitor of metalloproteinases or a nonspecific pharmaceutical MMP inhibitor, hinders angiogenesis[35-37], which we attribute to the prevention of gelatin formation by collagenases. Haptoglobin, in contrast, stimulates angiogenesis[30] which we attribute to inhibition of gelatinases without affecting collagenases. Our hypothesis may also explain why the increase of the MMP-9/TIMP balance failed to correlate with the migratory or invasive capacity of endothelial cells in vitro[38] because migration is also likely to depend on, besides on the MMP-9/TIMP balance, temporary matrix formation.

The amount and role of liver-derived plasma haptoglobin protein compared with artery-derived haptoglobin in the arterial wall is still unclear, as discrimination between haptoglobin produced by different tissues is not possible. High local arterial concentrations might be possible only when haptoglobin is produced at the site where it is needed. Another possibility is that arterial haptoglobin has other posttranslational modifications, such as glycosylation, compared with liver haptoglobin, resulting in another function or activity as shown for MD-2 and Toll-like receptor signaling[39].

In summary, our results demonstrate for the first time the expression of haptoglobin in adventitial fibroblasts of rabbit arteries after sustained changes in blood flow. Cell culture and in vitro assays showed that haptoglobin inhibited the breakdown of gelatin and promoted fibroblast cell migration. In vivo studies showed that arterial restructuring was delayed in haptoglobin knockout mice. We conclude that haptoglobin is involved in arterial restructuring by facilitating cell migration, probably via accumulation of a

temporary gelatin matrix. Promotion of cell migration by haptoglobin may be involved in other vascular and nonvascular processes such as angiogenesis, tissue repair, atherogenesis and tumor cell invasion.

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Nitric oxide synthesis is involved in arterial haptoglobin expression after sustained flow changes

Chapter III

M.B. Smeets, G. Pasterkamp, S.K. Lim, E. Velema, B. van Middelaar, D.P.V. de Kleijn

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Nitric oxide synthesis is involved in arterial haptoglobin expression after sustained flow changes.

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The acute phase protein haptoglobin is highly expressed in arteries after sustained flow changes and involved in cell migration and arterial restructuring. In the liver, haptoglobin expression is mainly regulated by interleukin-6 (IL-6). In the artery, shear stress and NO influence IL-6 expression. In the present study, we demonstrate that NO synthesis is involved in the regulation of arterial haptoglobin expression after sustained flow changes. Decreased haptoglobin expression after NO-inhibition coincided with decreased IL-6 levels. However, IL-6 knockout mice had normal arterial haptoglobin expression levels after sustained flow changes suggesting that other mediators may provide compensatory mechanisms for the regulation of arterial haptoglobin expression.

Introduction

Chronic changes in blood flow induce structural remodeling of the arterial wall to normalize shear stress[1]. Structural remodeling is an important determinant of luminal narrowing after balloon angioplasty and in de novo atherosclerosis. Previously, we demonstrated that arterial expression of the acute phase protein haptoglobin is increased during arterial remodeling after sustained flow changes and plays an important role in cell migration and arterial restructuring[2]. However, the regulatory pathways involved in arterial haptoglobin expression are unknown.

Liver haptoglobin expression is thought to be mediated through interleukin-6 (IL-6), rather than other inflammatory cytokines like glucocorticoids or TNFα. This is supported by point mutations in IL-6 responsive elements in the haptoglobin promoter region, resulting in lower serum haptoglobin levels[3-4]. Arterial IL-6 expression has been demonstrated in endothelial and smooth muscle cells during atherosclerosis[5-6], pointing to a regulatory role in arterial haptoglobin expression.

IL-6 expression in endothelial cells can be regulated by shear stress[7] and nitric oxide (NO)[8]. NO is an important mediator of arterial remodeling as is demonstrated by studies using either NO synthase (NOS) inhibitors[9] or endothelial NOS (eNOS)[10] and inducible NOS (iNOS)[11] knockout mice. NO mediates different processes that lead to arterial remodeling which involves the modulation of metalloproteinase (MMP) expression and activity[12] and cell migration[13]. Previous studies have shown that MMP inhibition can prevent constrictive remodeling and subsequent luminal narrowing[14-17].

We hypothesized that NO production after arterial flow changes regulates arterial expression of haptoglobin, a gelatinase inhibitor, through IL-6 expression. In this study, we show that NO synthesis is involved in the regulation of arterial haptoglobin expression after sustained flow changes and suggest that this NO-haptoglobin pathway is an important cellular mediator between sustained flow changes and arterial restructuring.

Material and methods

Animal models

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Rabbits: In 14 New Zealand White rabbits, the right carotid artery was completely ligated which also resulted in flow increase in the contralateral left carotid artery. Rabbits received either 1.5 g/liter L-NAME in the drinking water, starting 5 days before intervention (L-NAME treated group, n=7) or normal water (control group, n=7). The carotid arteries of 6 additional unoperated rabbits were used to obtain baseline expression levels. The rabbits were anaesthetized by intramuscular injection of methadone (0.15 ml) and ventraquil (0.15 ml) followed by intravenous injection of etomidate (1mg/kg) and ventilated with N_2O:O_2 and 0.6%

Halothane. Animals were sacrificed 1 day after operation and the carotid, femoral and iliac arteries were removed. The arteries were snap-frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction.
Mice: The IL-6 KO mice have been backcrossed into the wild type B6/129 background for at least 15 generations at the time of the experiments. Male wild type and IL-6 knockout mice (n=30) were anaesthetized using 0.05 ml/10 g body weight of a cocktail (1 part Hypnorm, 1 part Midazolam, 2 parts distilled water). The right common carotid artery was ligated as described by Kumar and Lindner[18]. Animals were sacrificed at 0 days (n=5/group) and 3 days (n=10/group) after operation and the left and right carotid arteries were removed. The arteries were snap-frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction.

RNA extraction
The frozen tissue samples were grinded in liquid nitrogen, using a pestle and mortar. RNA was extracted using 1 ml Tri-pure™ Isolation Reagent (Boehringer Mannheim) according to manufacturers protocol.

Northern blotting
Ten µg of RNA was separated on a 1.2% formaldehyde-agarose gel. After capillary blotting to Hybond-N membrane (Amersham), the membrane was baked for 2 hours at 80°C. Probes were prepared using the random primed DNA labeling kit (Boehringer Mannheim). Hybridization occurred for 1 hour at 65°C in Easy-Hyb hybridization solution (Stratagene) followed by 10 minutes washing in 0.1x standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) at 65°C. Bands were visualized using Biomax MS films (Kodak).

Quantitative real-time PCR
Reverse transcription was carried out with 500 ng total RNA using superscript II (Life) according to manufacturers protocol. To confirm the identity of the amplified cDNA products, PCR products were ligated into the pGEM®-T Easy Vector (Promega) and sequenced using the T7 sequenase version 2.0 DNA sequencing kit (Amersham).
PCR amplification was performed using the I-cycler iGTM Real Time PCR (Biorad). Each reaction contained 14 µl cDNA, 200 µM dNTP, 1x reaction buffer (Gibco-BRL) containing 1:80.0000 Cybergreen (Biorad), 2.5 U Taq DNA polymerase (Gibco-BRL) and 1 µM of each primer. Quantities were determined by comparison with known quantities of the cloned PCR product representing the target mRNA. Data were corrected for the amount of 18S mRNA that was used as an internal standard.
The following oligonucleotides were used as primers: rabbit haptoglobin (forward 5'-GAAGCAGTGGGTGAACAAGG-3', reverse 5'-TGAC AAGATTGTGGCGGGAG-3'), rabbit interleukin-6 (forward primer 5'-ACCACGATCCACTTCATCC-3', reverse primer 5'-TGTCCTAACG CTCATCTTC-3'), rabbit 18s (forward primer 5'-TCAACACGGGAAA CCTCAC-3', reverse primer 5'-ACAAATCGCTCCAGCAAC-3'), mouse haptoglobin (forward 5'-AAAAACCTCTTCCTGAACCAC-3', reverse 5'-AACGACCTTCTCAATCTCCAC-3'), mouse 18s (forward 5'-TCAACACGGGAAACCTCAC-3', reverse 5'-ACCAGACAAATC GCTCCAC-3').

Statistics
Data are presented as mean±sem. Statistical analysis was performed using the Mann-Whitney U test. P<0.05 was considered as statistically significant.

Results

To investigate the role of NO in the regulation of arterial haptoglobin expression after flow changes in rabbit carotid arteries, NO synthesis was inhibited using the nonspecific NOS inhibitor L-NAME. Northern blot analysis showed that after flow changes, haptoglobin mRNA levels increased more in carotid arteries of control rabbits compared to the carotid arteries of L-NAME treated rabbits, 1 day after flow changes (figure 1A).
Quantitative real-time PCR was used to quantify the relative amounts of haptoglobin mRNA levels in the carotid arteries of unoperated, control and L-NAME treated rabbits. In control rabbits, both a flow increase and flow decrease in the carotid artery resulted in a significant increase of arterial haptoglobin mRNA expression compared to baseline values (30-fold, p=0.005; figure 1B). In L-NAME treated rabbits, a significant increase in haptoglobin mRNA levels was observed compared to baseline values (12-fold, p=0.005).

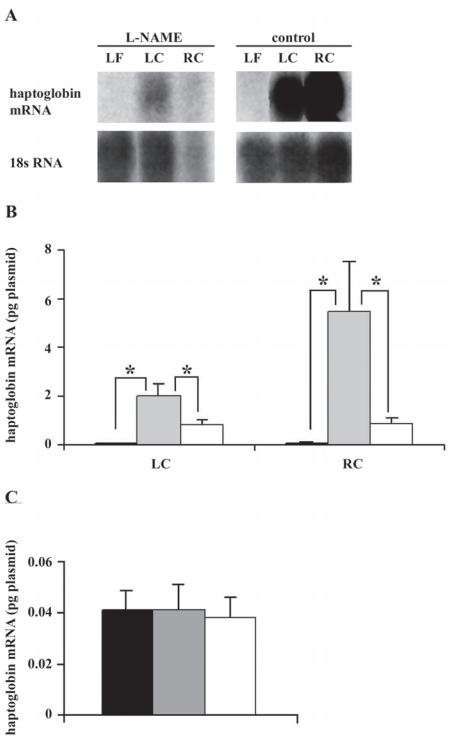

Figure 1. Arterial haptoglobin mRNA expression in rabbit carotid arteries after L-NAME treatment, 1 day after sustained flow changes
A) Northern blot hybridization showing haptoglobin mRNA expression in L-NAME treated and control rabbits, 1 day after flow changes. LF=unoperated left femoral artery; LC=left carotid artery; RC=right carotid artery. **B)** Haptoglobin mRNA expression in carotid arteries after sustained flow changes. **C)** Haptoglobin mRNA expression in unoperated femoral and iliac arteries. Haptoglobin mRNA is presented as the amount of plasmid containing the haptoglobin PCR product to which it correlates in the dilution series of this plasmid used in the quantitative real-time PCR. n=7 rabbits/group, * p<0.05; black bar=baseline values; grey bar=control rabbits; white bar=L-NAME treated rabbits.

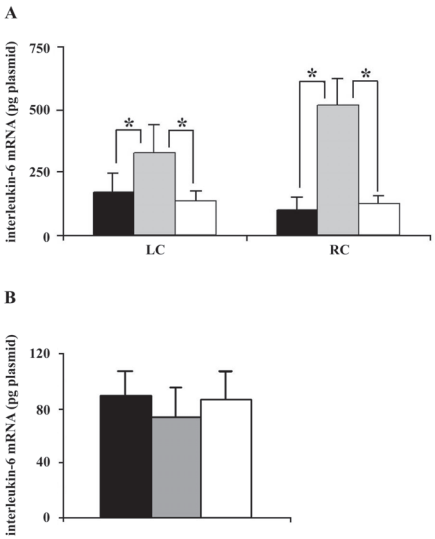

Figure 2. Interleukin-6 mRNA expression in rabbit carotid arteries after L-NAME treatment, 1 day after sustained flow changes
IL-6 mRNA is presented as the amount of plasmid containing the IL-6 PCR product to which it correlates in the dilution series of this plasmid used in the quantitative real-time PCR. **A)** IL-6 mRNA expression in carotid arteries after sustained flow changes. **B)** IL-6 mRNA expression in unoperated femoral and iliac arteries. n=7 rabbits/group, * p=0.04; LC=left carotid artery; RC=ligated right carotid artery; black bar=baseline values; grey bar=control rabbits; white bar=L-NAME treated rabbits.

Absolute haptoglobin mRNA levels, however, were significantly decreased in the carotid arteries of L-NAME treated rabbits compared to control rabbits (33%, p=0.04; figure 1B). Basal haptoglobin mRNA expression in unoperated femoral and iliac arteries was not affected by L-NAME treatment (figure 1C).
IL-6 is thought to be the major stimulator of haptoglobin expression. Therefore, we investigated if IL-6 expression decreased after L-NAME treatment. One day after flow changes, IL-6 mRNA levels were significantly increased compared to baseline values in both the left and right carotid artery of untreated rabbits (4-fold, p=0.03; figure 2A). However, no increase in IL-6 expression levels was observed in the carotid arteries of L-NAME treated rabbits compared to baseline values. L-NAME treatment had no effect on basal expression levels of IL-6 mRNA in unoperated femoral and iliac arteries (figure 2B).

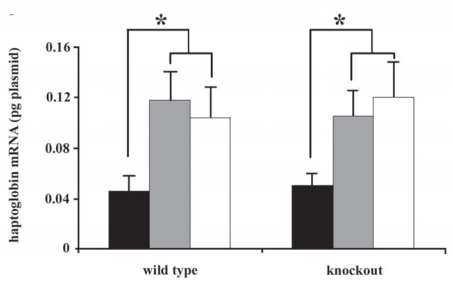

Figure 3. Haptoglobin mRNA expression in carotid arteries in IL-6 knockout mice after sustained flow changes
Haptoglobin mRNA is presented as the amount of plasmid containing the haptoglobin PCR product to which it correlates in the dilution series of this plasmid used in the quantitative real-time PCR. n=8-10 arteries/group, * p=0.05; black bar=carotid arteries, 0 days; grey bar=left carotid artery 3 days after ligation; white bar=ligated right carotid arteries, 3 days after ligation.

To confirm the possible role of IL-6 in arterial haptoglobin expression, haptoglobin expression was measured in IL-6 knockout mice following ligation of the right carotid artery (figure 3). In wild type mice, haptoglobin mRNA levels increased in the left and right carotid artery at 3 days after ligation of the right carotid artery (2-fold, p=0.05). A similar increase in haptoglobin mRNA expression was observed in IL-6 knockout mice 3 days after ligation of the carotid artery (2-fold, p=0.05). Baseline haptoglobin expression levels did not differ between wild type and IL-6 knockout mice (figure 3).

Discussion

Haptoglobin expression is increased in arteries after sustained flow changes and involved in cell migration and arterial restructuring[2]. The arterial response to normalize wall shear stress after blood flow changes depends on a functional vascular endothelium[19] that releases NO as a mediator in the arterial remodeling process. In the present study, we used the arterial ligation model to investigate if NO induces arterial haptoglobin expression after flow changes.
Inhibition of NO after flow changes using the non-specific NOS inhibitor L-NAME partly inhibited arterial haptoglobin expression. This shows that a NO-dependent pathway is involved in the regulation of arterial haptoglobin expression after flow changes. There are at least two isoforms of NOS expressed in the vascular system after

injury or flow changes[20-22]. There are, however, differences in function between those isoforms. NO derived from eNOS inhibits neointimal formation whereas NO derived from iNOS promotes neointimal formation. Moreover, constrictive remodeling is impaired in eNOS knockout mice whereas iNOS knockout mice display more constrictive remodeling compared to wild type mice[10-11, 23]. Unfortunately, the use of L-NAME did not allow for identification of the NOS isoform that is involved in the regulation of arterial haptoglobin expression.

There are no reports demonstrating a direct effect of NO or shear stress on haptoglobin expression. However, some of the genes which are regulated by NO appear to depend on the presence of diffusible intermediates like cytokines[24]. IL-6 expression is an important regulator of haptoglobin expression and expressed in both endothelial[7-8] and smooth muscle cells[5-6] during atherosclerosis. Moreover, IL-6 expression can be regulated by NO and shear stress[7-8], making it a potential candidate to function as an intermediate between increased NO synthesis and arterial haptoglobin expression.

To investigate a possible regulatory role for IL-6 in arterial haptoglobin expression, we measured IL-6 expression levels after L-NAME treatment. Although the L-NAME treated rabbits still responded to flow changes with increased haptoglobin mRNA levels, there was a significant decrease in the absolute levels of haptoglobin mRNA. This decrease in haptoglobin coincided with baseline levels of IL-6, supporting a regulatory role for IL-6 in arterial haptoglobin expression.

IL-6 knockout mice were used to confirm if arterial haptoglobin expression indeed is regulated through IL-6 after sustained flow changes. Surprisingly, the IL-6 knockout mice responded normal to flow changes with increased haptoglobin mRNA levels, demonstrating that IL-6 is not the only regulator for the induction of haptoglobin in the arterial wall. This is in accordance with other studies showing that IL-6 is not the sole regulator of haptoglobin expression[25]. For instance, the induction of serum haptoglobin in IL-6 and IL-6/TNF/LTalpha knockout mice is only slightly reduced after stimulation with lipopolysaccharide (LPS)[26]. Apparently, multiple signals exist which are able to induce haptoglobin expression and can compensate for deficiencies in regulators like IL-6.

In summary, this study demonstrates that NO synthesis is involved in arterial expression of haptoglobin after sustained flow changes, coinciding with increased IL-6 levels. Since the increase in haptoglobin expression is normal in IL-6 knockout mice after flow changes, we infer that other unidentified mediators may provide compensatory mechanisms for the regulation of arterial haptoglobin expression after flow changes.

Acknowledgements

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III





The acute phase protein haptoglobin is locally expressed in arthritic and oncological tissues

Chapter IV

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The acute phase protein haptoglobin is locally expressed in arthritic and oncological tissues.

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Haptoglobin is an acute phase protein known to be highly expressed in the liver. Recently, we showed increased local arterial haptoglobin expression after flow-induced arterial remodelling and found that haptoglobin is involved in cell migration and arterial restructuring probably through accumulation of a temporary gelatine matrix. Since cell migration and matrix turnover are important features in the pathology of arthritis and cancer, we hypothesised that haptoglobin is also expressed in arthritic and oncological tissues. In this study, we investigated local haptoglobin expression in arthritic rats (n=12) using semi-quantitative PCR, western blotting and studied haptoglobin mRNA localisation in human kidney tumours (n=3) using *in situ* hybridisation. The arthritic rats demonstrated an increase of haptoglobin mRNA (2.5-fold, p<0.001) and protein (2.6-fold, p<0.001) in the arthritic achilles tendon. Haptoglobin protein was also increased in the arthritic ankle (2.6-fold, p<0.001) but not in the non-arthritic knee. In human kidney tumours, tumour and stromal cells produced haptoglobin mRNA. This study shows that the liver protein haptoglobin is, besides the artery, also expressed in arthritic and oncological tissues recognised for enhanced cell migration and matrix turnover.

Introduction

Haptoglobin is mainly produced in the liver but expression can also be induced in various other tissues[1]. Recently, we demonstrated that the acute phase protein haptoglobin is a natural inhibitor of collagen degradation and is locally expressed in fibroblasts of the arterial wall. Haptoglobin plays an important role in cell migration and arterial restructuring[2]. Collagen turnover is an important feature in many physiological processes like growth and wound healing. Enhanced collagen degradation is observed and often causally related with severe tissue destruction or malfunction as can be seen in the pathological processes of arthritis[3] and cancer[4].

The importance of haptoglobin in cell migration and extracellular matrix degradation suggests a role for haptoglobin in arthritis and cancer. Both disease processes are characterised by increased cell migration and degradation of the extracellular matrix. Invasion of arthritic fibroblast-like synoviocytes rapidly destroys a cartilage matrix[5]. In cancer, the migration of cells plays a role in tumor angiogenesis, progression and metastasis[6].

Although sero-epidemiological studies reported increased haptoglobin serum levels during arthritis and carcinogenesis[7-8], local expression of haptoglobin in arthritic and oncological tissues has not been studied before. We hypothesised that local expression of haptoglobin will increase in arthritic and oncological tissues in which cell migration and matrix remodelling are important features.

In the present study, we investigated local expression of haptoglobin in arthritis and cancer and describe that haptoglobin was locally expressed in arthritic and oncological tissues in which extracellular matrix turnover and cell migration are predominant features.

Material and methods

Tissue material

Arthritic rats: In 6-8 weeks old inbred male Lewis rats (n=6), arthritis was induced by one single intradermal injection of 5 µg/ml heat-killed Mycobacterium tuberculosis (strain H37Ra, Difco) in Freund's incomplete adjuvant (Difco) in the base of the tail[9]; 6 additional rats were used as control. The rats were terminated after 6 weeks. The arthritic ankle and Achilles tendon as well as the unaffected knee were removed, snap-

frozen in liquid nitrogen and stored at –80°C for RNA and protein extraction.
All investigations conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No.85-23, 1985) and were approved by the ethical committee on animal experiments of the University Medical Center Utrecht.
Human kidney tumours: Surgical specimens from human Grawitz kidney tumours were immediately snap-frozen in liquid nitrogen and stored at -80°C for *in situ* hybridisation (n=3).

RNA and protein extraction
The frozen tissue samples were grinded in liquid nitrogen, using a pestle and mortar. RNA and protein were extracted using 1 ml Tri-pure™ Isolation Reagent (Boehringer Mannheim) according to manufacturer's protocol.

Semi-quantitative RT-PCR
Specific sets of primers were constructed using software at CMBI (Nijmegen). Reverse transcription was carried out with 500 ng total RNA using superscript II (Life) according to manufacturers protocol. To confirm the identity of the amplified cDNA products, PCR products were ligated into the pGEM®-T Easy Vector (Promega) and sequenced using the T7 sequenase version 2.0 DNA sequencing kit (Amersham). PCR amplification on arthritic rats was performed using 11 µl cDNA, 200 µM dNTP, 1x reaction buffer (BRL), 2.5 U Taq DNA polymerase (BRL) and 1 µM of each primer. The reaction was run on 8% polyacrylamide gels, stained with ethidium bromide and analysed using 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 no genomic DNA was present (data not shown). Data were corrected for the amount of β-actin mRNA, which was used as an internal standard. The following oligonucleotides were used as primers: rat haptoglobin (forward primer 5'-TGATCAAGCTCAAACAGAAAGTG-3', reverse primer 5'-ATAGCAAGTGTCTTCCTCATACTT-3'), rat β-actin (forward primer 5'-AAGGAACAACCCAGCATCC-3', reverse primer 5'-AGAGCCACCAATCCACACA-3').

In situ hybridisation
Human haptoglobin cDNA in pGEM®-T Easy Vector was linearised and used as template to obtain Digoxigenin (DIG, Roche) labelled RNA probes according to manufacturers protocol. Tissue segments were cut into 8 µm sections and transferred to Superfrost plus slides (Menzel Glazer) and stored at –80°C until use.
After defrosting, sections were treated with 0.2 M HCl for 20 min at RT, washed three times with PBS for 5 min and treated with proteinase K (Roche 10 µg/ml) for 10-15 minutes at 37°C in PBS. The sections were washed with PBS and fixed at RT for 5 minutes in 4% paraformaldehyde and treated twice for 5 min with acetic anhydride in triethylacetate (TEA, 185 µl acetyl anhydride in 0.1 M TEA). Sections were subsequently washed twice in 2xSSC for 5 min at RT, followed by 5 min in 2XSSC/ 50%formamide at 37°C.
For prehybridisation, 100 µl hybridisation mix (50% formamide, 1mg/ ml tRNA, 1x Denhardts, 10% dextrane sulfate, 4xSSC) was added to the slide and incubated for 1 hour at 46°C. After °/n hybridisation with a DIG (1 µl)-labeled probe at 46°C, sections were washed with 0.1xSSC at 45°C for 15 min, followed by RNase treatment (40 µg/ml RNase A, 1mM EDTA pH8.2, 2xSSC) for 15 min at RT and washed again with 0.1xSSC at 45°C for 15 min. Before detection with 1/500 sheep-α-DIG AP (Boehringer), sections were rinsed with 2xSSC and 100 mM Tris pH7.4-150 mM NaCl at RT. Detection with NCBI/NBT (Roche) was performed according to manufacturer's protocol.

Western blotting
Equal amounts of protein (5 µg/lane) were separated on a 10% SDS polyacrylamide gel and blotted onto Hybond ECL (Amersham) in blotting buffer (14.4 g/l Glycine, 3.03 g/l Tris, 20% methanol). The membrane was blocked °/n in PBS-0.1% Tween-5% non-fat dry milk (PBSTP). The membrane was incubated for 1 hour at RT with 1/4000 goat-α-human haptoglobin (ICN), followed by 1/1000 biotinylated rabbit-α-goat and 1/2000 streptavidine-horseradish peroxidasae. All incubation steps were performed in PBSTP and subsequently washed 3 times in PBS-0.1% Tween (PBST). Detection occurred using ECL (NEN Life Science products) and exposure to X-Omat Blue XB-1 films (Kodak).

Statistics
Data are presented as mean±sem. The arthritic rats were analysed using the Mann-Whitney U test. P values <0.05 were considered as statistically significant.

Results

Local haptoglobin expression in arthritic tissue.

Haptoglobin expression in the arthritic rats (n=12) was investigated using the affected ankle and achilles tendon and the unaffected knee. Haptoglobin mRNA expression was 2.5-fold increased in the achilles tendon in arthritic rats ($p<0.001$) (Figure 1A). Haptoglobin mRNA levels in the ankle revealed large fluctuations within the two groups but showed no significant differences between the arthritic and control rats ($p=0.51$) (figure 1A). No significant difference was found in haptoglobin mRNA levels in the

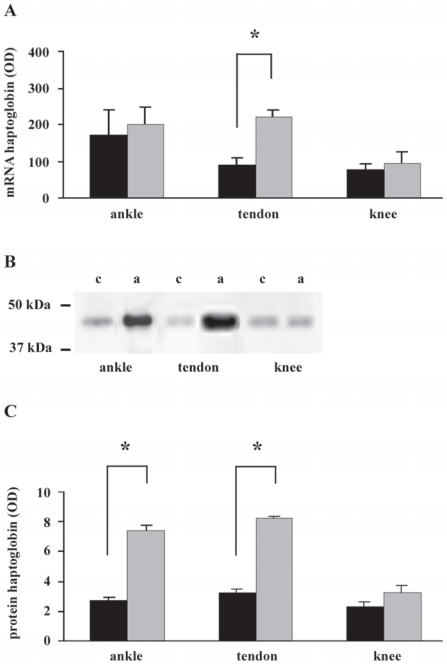

Figure 1. Haptoglobin mRNA and protein expression in arthritic rats
A) haptoglobin mRNA levels in control and arthritic rats, **B)** western blot analysis of haptoglobin protein in ankle, tendon and knee (c=control rat, a=arthritic rat), **C)** haptoglobin protein levels in control and arthritic rats. n=9, * p<0.001, black bar=control rats, grey bar=arthritic rat.

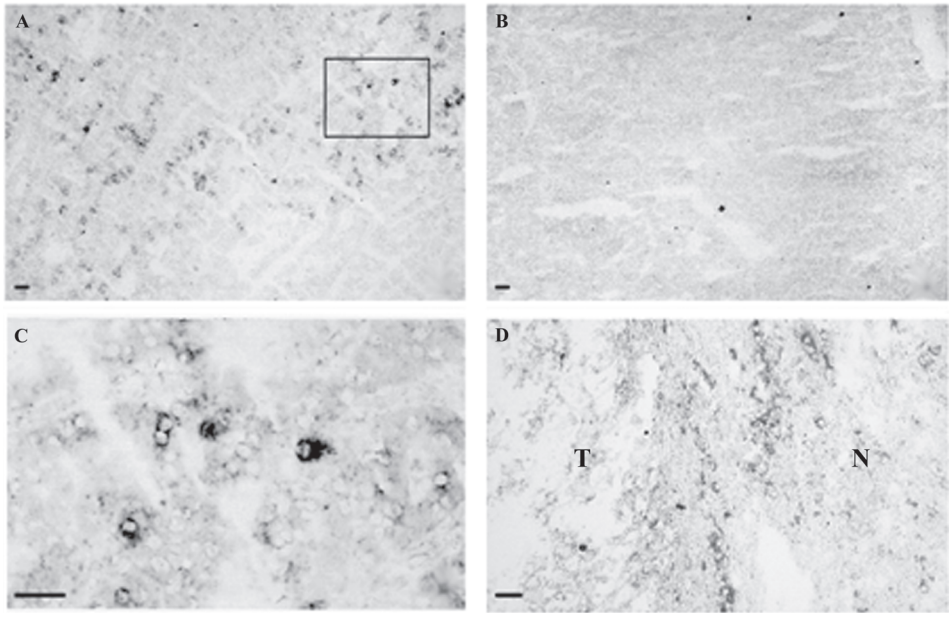

Figure 2. *In situ* hybridisation on human kidney tumours using an anti-sense probe against human haptoglobin mRNA
A) haptoglobin mRNA expression by invasive tumour cells (magnification=100x), **B)** *in situ* hybridisation using a sense probe (magnification=100x), **C)** detail of the indicated area in A (magnification=400x) **D)** haptoglobin mRNA expression by surrounding stromal cells (magnification=200x). T= tumour tissue, N=normal kidney tissue, bar=25 µm.

unaffected knee between arthritic and control rats. Western blotting revealed that haptoglobin protein expression was 2.6-fold increased in both the arthritic ankle and the arthritic Achilles tendon ($p<0.001$) from arthritic rats (figure 1B +C). No differences in haptoglobin protein expression were found in the unaffected knee.

Haptoglobin expression in oncological tissue.
In situ hybridisation on human Grawitz tumours (n=3) demonstrated haptoglobin expression by tumour cells located in invasive parts of the tumour (figure 2A+C). Alternate sections hybridised with a control sense probe showed no staining (figure 2B). Interestingly, positive staining was also found in surrounding stromal tissue (figure 2D) by macrophages and fibroblast-like cells (data not shown).

Discussion

Cell migration and collagen degradation are key events in arthritis[3, 10], tumour progression and tumour metastasis[4, 6]. Enhanced degradation of collagen is required for cell migration to occur[11-12]. Recently, we have identified haptoglobin as an essential factor for cell migration[2]. Haptoglobin knockout cells demonstrated impaired migration that could be restored by supplementation of exogenous haptoglobin to the cells. Although serum haptoglobin levels have previously been associated with the progression and outcome of arthritis and cancer[7-8], local haptoglobin synthesis has not been studied before in these pathological tissues. As cell migration is believed to be a tightly controlled process that depends on matrix degradation in the immediate surrounding of cells[13], we investigated in the present study the local expression of haptoglobin in arthritic and oncological tissues. Previous studies have demonstrated increased haptoglobin levels in the serum during arthritis[7]. In arthritic rats, the affected ankle and Achilles tendon showed significantly increased levels of local haptoglobin expression. No differences were found in the unaffected knee demonstrating that increased haptoglobin protein levels in the affected joints are the result of local haptoglobin expression and not due to extravasation of

haptoglobin from the serum.
Fibroblast-like synoviocytes of human arthritic tissue migrate *in vivo* into cartilage matrix resulting in a destructive pannus as is found in human arthritic tissue[5]. These fibroblast-like synoviocytes are the main producers of the high local levels of MMPs[10, 14] that correlate with the severity of the lesion and are mainly produced in arthritic tissue[10]. We infer that local haptoglobin expression facilitates cell migration in the cartilage and may therefore play a role in the progression of arthritis.
Haptoglobin mRNA was also locally expressed in kidney tumour tissue. Cells staining positive for haptoglobin mRNA were mainly found in the invasive part of the tumour and in the surrounding stroma. Haptoglobin protein levels were not measured in the tumour samples due to contamination with blood residues. The localisation of haptoglobin correlated with the earlier described expression pattern of MMP-2, which is mainly synthesised by fibroblasts in the stroma surrounding the tumours although the active protein can also be found around tumour cells[15-16]. Furthermore, MMP synthesis by surrounding stromal cells is necessary for tumour metastasis indicating the important role of stromal-derived MMP activity[17]. Increased expression of MMPs has been associated with tumour invasion, metastasis and angiogenesis and the controlled degradation of surrounding stromal matrix appears to be essential for these three processes[15-16, 18]. We suggest that haptoglobin provides tumour cells with an additional level of MMP-2 regulation besides the controlled activation via MT1-MMP[19-20] thereby facilitating tumour cell invasion and metastasis.
Elevations in serum haptoglobin levels during pathological processes have been demonstrated by various sero-epidemiological studies. However, local expression of haptoglobin in pathological tissues has not been studied. There are two possible explanations for the necessity of increased local haptoglobin synthesis in pathological processes, despite the normal presence of haptoglobin in the serum. First, increased local expression might be the only way to achieve high local concentrations of haptoglobin which are necessary for the inhibition of local gelatinase activity. Second, local production of haptoglobin might reflect the synthesis of a functionally distinct protein, compared to haptoglobin produced in the liver.
It is known for several proteins like eNOS or Toll-like receptor-4 that changes in the amount and nature of glycosylation can alter protein function drastically[21-22]. Haptoglobin is a protein with several posttranslational glycosylations on the β-chains[23] and alterations in the glycosylation pattern of haptoglobin have been described during the development and progression of various pathological processes and even appear to have prognostic values for some diseases[24]. Increased local haptoglobin expression with other glycosylation patterns might therefore reflect an altered function compared to haptoglobin produced in the liver.
A limitation of this study is that it is purely descriptive and we can only speculate about the exact function of haptoglobin in these tissues. However, haptoglobin is locally produced in both pathological tissues and is described to be involved in cell migration and matrix degradation. Since cell migration and matrix degradation are important features in arthritis and cancer, this suggests a local role for haptoglobin in these processes and supports a role for haptoglobin in the initial response to tissue injury. This is in accordance with studies in haptoglobin knockout mice that showed a delayed response to arterial[2] or kidney injury[25].
In summary, we have investigated local haptoglobin expression in arthritis and cancer where cell migration and matrix remodelling are important features and describe an upregulation of local haptoglobin expression in arthritic and oncological tissues.

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We thank Elianne Koop for providing tissue samples of human kidney tumours and Roel Goldschmeding for the evaluation of the human kidney tumours. This study was supported by grants from the Netherlands Organisation for Scientific Research (NWO 902-16-239 and 902-16-222) and the Netherlands Heart Foundation (99-209).

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IV





Increased arterial expression of a novel glycosylated haptoglobin isoform after balloon dilation

Chapter V

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Submitted

Increased arterial expression of a novel glycosylated haptoglobin isoform after balloon dilation.

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Background: Haptoglobin is an acute phase glycoprotein that is highly expressed in the liver and secreted into the serum. Recently, we observed arterial haptoglobin expression after sustained flow changes and found that haptoglobin is a cell migration factor involved in arterial restructuring. Although the function of extrahepatic haptoglobin expression is not yet understood, local haptoglobin expression may provide the tissue with functionally different haptoglobin due to posttranslational modifications. We hypothesized that haptoglobin expression is increased during arterial restructuring after balloon dilation and compared glycosylation patterns between arterial and liver haptoglobin. Methods and Results: Arterial haptoglobin expression was studied in rabbits at 0, 2, 7, 14 and 28 days after balloon dilation (n=36) using quantitative PCR, western blotting and in situ hybridization. After balloon dilation, arterial haptoglobin mRNA levels were maximally increased at 7 days (5.7-fold, p=0.01), whereas protein levels were maximal at 14 days (1.4-fold, p=0.01). Haptoglobin was only expressed in the adventitia of balloon dilated arteries, which was confirmed in human atherosclerotic coronary arteries. Liver haptoglobin expression remained constant after balloon dilation. Two-dimensional gel electrophoresis and lectin affinity blotting were used to identify liver and arterial haptoglobin glycoforms and revealed the expression of artery-specific haptoglobin glycoforms. Conclusions: This study demonstrates that arterial haptoglobin expression is increased early after balloon dilation whereas liver haptoglobin expression did not change. Furthermore, arterial haptoglobin consists of an unique set of glycoforms compared to haptoglobin produced in the liver.

Introduction

Haptoglobin is an acute phase glycoprotein that is mainly produced in the liver and secreted into the serum[1-2]. There is, however, increasing evidence that haptoglobin is also expressed in various extrahepatic tissues like lung, kidney, skin and heart[3-4]. Furthermore, extrahepatic haptoglobin expression can be induced by lipopolysaccharide (LPS)[5].

Recently, we demonstrated that haptoglobin is locally expressed in the arterial wall after sustained flow changes. Haptoglobin was found to play an important role during cell migration and involved in flow-induced arterial restructuring[6]. Arterial restructuring determines the degree of lumen loss in pathological arterial processes like restenosis[7] and atherosclerosis[8], suggesting a role for haptoglobin in these processes.

Although the function of extrahepatic haptoglobin synthesis is not yet understood, local haptoglobin synthesis may provide tissues with a source of functionally or structurally different haptoglobin. Tissue-specific glycosylation of proteins has been demonstrated for many proteins and it is known that glycosylation can modify protein function or structure[9]. Serum haptoglobin concentrations increase during inflammation[10] and tissue injury[11] and are accompanied by changes in haptoglobin glycosylation that have been associated with disease development and progression[12]. Thus, increased extrahepatic synthesis and secretion of differentially glycosylated haptoglobin might

explain the observed variations in haptoglobin glycosylation during various diseases.
In this study, we hypothesize that arterial haptoglobin synthesis is increased after balloon dilation and characterized by differentially glycosylated arterial haptoglobin compared to liver derived haptoglobin. We show that arterial haptoglobin expression is increased in the adventitial layer early after balloon dilation in rabbits whereas haptoglobin liver expression remains constant. Furthermore, we demonstrate that arteries produce an unique set of haptoglobin glycoforms that is distinctive from liver derived haptoglobin.

Material and Methods

Tissue material
Rabbits Balloon dilation was performed in the femoral and iliac arteries of 30 New Zealand white rabbits. The contra-lateral artery was used as a control artery. Undilated femoral and iliac arteries from six additional rabbits were used to determine haptoglobin baseline levels (=day 0). The rabbits were anesthetized by intramuscular injection of methadone (0.15 ml) and vetranquil (0.15 ml) followed by intravenous injection of etomidate (1mg/kg) and ventilated with N_2O:O_2 and 0.6% Halothane. A 3.0 mm balloon was inflated 3x30 seconds with a pressure of 6-8 atmospheres. Arterial diameters changes at the internal elastic lamina were determined by measuring arterial lumen using angiography after balloon dilation and at termination. Arterial diameter changes were corrected for intimal hyperplasia thickness determined by histology cross-sectional analysis. After 0, 2, 7, 14 and 28 days, the rabbits were sacrificed. A small segment of the artery was fixated for 2 hours in 4% paraformaldehyde at room temperature (RT), followed by over night (%) incubation in 15% sucrose in PBS. After embedding in Tissue Tec (Sakura), the segment was stored at -80°C. The remaining part of the artery was snap-frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction. Liver samples were snap-frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction. Blood samples were collected at termination; serum was isolated after centrifugation and stored at -80°C.
All investigations conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No.85-23, 1985) and were approved by the ethical committee on animal experiments of the University Medical Center Utrecht.
Human coronary arteries Coronary arteries (left anterior descending, left circumflex and right coronary arteries), removed directly after heart transplantation, were used to perform in situ hybridization (n=3). The arteries were fixated for 2 hours in 4% paraformaldehyde at RT, followed by % incubation in 15% sucrose in PBS. After embedding in Tissue Tec (Sakura), the segment was stored at -80°C.

RNA and protein extraction
The frozen tissue samples were grinded in liquid nitrogen, using a pestle and mortar. RNA and protein were extracted using 1 ml Tri-pure™ Isolation Reagent (Boehringer Mannheim) according to manufacturers protocol. Protein samples for 2D gel electrophoresis were isolated using 40 mmol/L Tris, pH7.5. Protein concentrations were determined using the Bio-rad DC protein assay.

Real-time PCR
Reverse transcription was carried out with 500 ng total RNA using superscript II (Life) according to manufacturers protocol. To confirm the identity of the amplified cDNA products, PCR products were ligated into the pGEM®-T Easy Vector (Promega) and sequenced using the T7 sequenase version 2.0 DNA sequencing kit (Amersham).
PCR amplification was performed using the I-cycler iGTM Real Time PCR (Bio-rad). Each reaction contained 14 µl cDNA, 200 µM dNTP, 1x reaction buffer (BRL) containing 1:100.0000 Cybergreen (Bio-rad), 2.5 U Taq DNA polymerase (BRL) and 1 µM of each primer. Quantities were determined by comparison with known quantities of the cloned PCR products representing the target mRNAs. Data were corrected for the amount of 18S or β-actin mRNA, which were used as internal standards.
The following oligonucleotides were used as primers: rabbit haptoglobin (forward primer 5'-GAAGCAGTGGGTGAACAAGG-3', reverse primer 5'-TGACAAGATTGTGGCGGGAG-3'), rabbit 18s (forward primer 5'-TCAACACGGGAAACCTCAC-3', reverse primer 5'-ACAAATCGCTC CAGCAAC-3'), human haptoglobin (forward primer 5'-TTCTACAC CCTAACTACTCCCAGG-3', reverse primer 5'-TAACCCACAC GCCC TACTTC-3'), human β-actin (forward primer 5'-CTGTACGCCAAC ACAGTG CT-3', reverse primer 5'TCCACACAGAGTACTTGCGC-3').

In situ hybridization
Human and rabbit haptoglobin cDNA in pGEM®-T Easy Vector was linearized and used as template to obtain digoxigenin (DIG, Roche) labeled RNA probes according to manufacturer's protocol. Tissue segments were cut into 8 µm sections and transferred to Superfrost plus slides (Menzel Glazer) and stored at –80°C until use.
After defrosting, sections were treated with 0.2 mol/L HCl for 20 min at RT, washed three times with PBS for 5 min and treated with proteinase K (Roche 10 µg/ml) for 10-15 minutes at 37°C in PBS. The sections were washed with PBS and fixed at RT for 5 minutes in 4% paraformaldehyde and treated twice for 5 min with acetic anhydride in triethylacetate (TEA, 185 µl acetyl anhydride in 0.1 mol/L TEA). Sections were subsequently washed twice in 2xSSC for 5 min at RT, followed by 5 min in 2XSSC/50% formamide at 37°C.
For prehybridization, 100 µl hybridization mix (50% formamide, 1mg/ml tRNA, 1x Denhardts, 10% dextrane sulfate, 4xSSC) was added to the slide and incubated for 1 hour at 46°C. After % hybridization with a DIG (1 µl)-labeled probe at 46°C, sections were washed with 0.1xSSC at 45°C for 15 min, followed by RNAse treatment (40 µg/ml RNAse A, 1mmol/L EDTA pH8.2, 2xSSC) for 15 min at RT and washed again with 0.1xSSC at 45°C for 15 min. Before detection with 1/500 sheep-α-DIG AP (Boehringer), sections were rinsed with 2xSSC and 100 mmol/L Tris pH7.4+150 mmol/L NaCl at RT. Detection with NCBI/NBT (Roche) was performed according to manufacturer's protocol.

One- and two-dimensional gel electrophoresis
For one-dimensional gel electrophoresis, equal amounts of total protein (5 µg/lane) were separated on a 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) under reducing conditions.
For two-dimensional gel (2D gel) electrophoresis, equal amounts of protein (100 µg for tissue samples, 1 µl for serum) were diluted to 125 µl with rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% Bio-Lyte® 3/10 ampholyte, 0.001% bromophenol blue; Bio-rad) and used to rehydrate 7 cm IPG dry strips pH4-7 (Bio-rad) at 50 Volt for 12 hours at 20°C. The rehydrated strips were focussed by 2 hours ramping to 4000 Volt, followed by an additional 20,000 Volthours. The strips were placed for 10 minutes in equilibration solution (6 M urea, 375 mM Tris-HCl pH8.8, 30% glycerol, 2% SDS; Bio-rad) containing 100 mM DTT, followed by 10 minutes in equilibration buffer containing 135 mM iodoacetamide. The equilibrated strips were run on a 12% SDS-PAGE.

Western blotting and lectin affinity blotting
Proteins were electrophoretically transferred onto Hybond ECL (Amersham) and blocked % at 4°C in block solution (PBS, 0.1% Tween, 5% non-fat dry milk). The membrane was incubated for 1 hour at RT with goat-α-human haptoglobin (Sigma, 1/1000), biotinylated GNA (EY laboratories, 1/1000) or biotinylated lotus tetragonolobus (Sigma, 1/1000), followed by 1 hour incubation with biotinylated rabbit-α-goat (DAKO, 1/1000) and peroxidase labeled streptavidine (SBA, 1/1000). Detection occurred using ECL (NEN Life Science products) and exposure to X-Omat Blue XB-1 films (Kodak). As a negative control, the goat-α-human haptoglobin antibody was substituted by normal goat serum or blocked with 4 µg purified human serum haptoglobin (Sigma).

Statistics
Data are presented as ratio operated versus control artery or mean±sd. Statistical analysis for the balloon dilated rabbits was performed using a Wilcoxon matched pairs signed rank sum test. P values <0.05 were considered as statistically significant.

Results

Haptoglobin expression after balloon dilation

The arterial response after balloon dilation is depicted in figure 1A. At 2 days after balloon dilation, the balloon dilated arteries were slightly enlarged. Constrictive remodeling started at day 7 after balloon dilation and continued up to 28 days. Neointima formation was first found at 7 days after balloon dilation and progressively continued up to 28 days. Since both arteries reacted the same to balloon dilation, the data from femoral and iliac arteries were pooled.

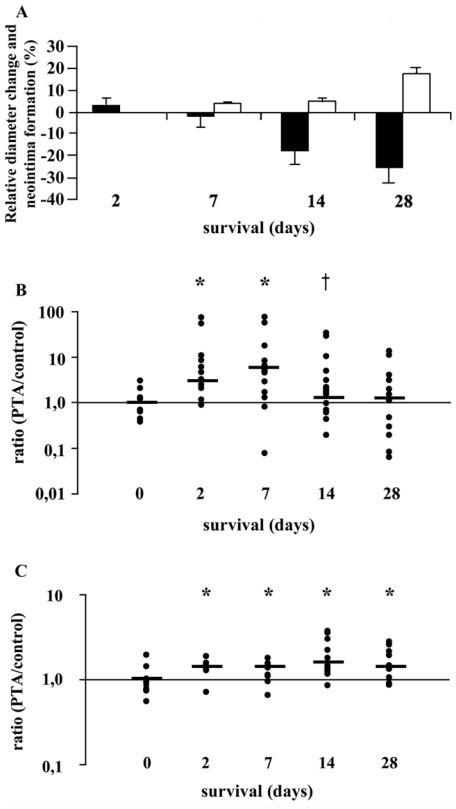

Figure 1. Haptoglobin mRNA and protein levels after balloon dilation in rabbit femoral and iliac arteries
A) mean intimal hyperplasia (as % of total arterial diameter, white bars) and the relative diameter change (as % of total arterial diameter, black bars) in balloon dilated arteries between post-dilation and at termination, **B)** ratio of haptoglobin mRNA levels between balloon dilated and control arteries, and **C)** ratio of haptoglobin protein between balloon dilated and control arteries. n=6-8 rabbits per time-point, * p=0.01, † p=0.04.

To measure haptoglobin mRNA and protein expression, real-time PCR and western blotting was performed. Haptoglobin mRNA expression was significantly increased at 2, 7 and 14 days ($p<0.04$) after balloon dilation with peak values at 7 days (median=5.7-fold) (figure 1B). At 28 days, haptoglobin mRNA levels were returned to baseline levels. Haptoglobin protein levels were significantly increased in the balloon dilated arteries with a maximum at 14 days after balloon dilation (median=1.4-fold, p=0.01) (figure 1C). The signal disappeared after blocking the primary haptoglobin antibody with an excess of purified haptoglobin or substitution with normal goat serum (data not shown).
To identify the cells expressing haptoglobin, we performed in situ hybridization. A strong staining for haptoglobin mRNA in balloon dilated rabbit arteries was observed in cells in the adventitial layer (figure 2A); no staining was observed in the media or neointima. The localization of haptoglobin mRNA in rabbit arteries was confirmed by in situ hybridization in human atherosclerotic coronary arteries where haptoglobin mRNA was mainly expressed in adventitial cells although some positive cells were also found in the media and plaque (figure 2C). Alternate sections hybridized with a control sense probe showed no staining (figure 2B+D).

Arterial versus liver haptoglobin expression

To investigate the relative importance of arterial haptoglobin synthesis, haptoglobin mRNA and protein levels were compared before and 2 days after balloon dilation in liver and arterial tissue samples. Haptoglobin mRNA and protein levels increased significantly in the artery after balloon dilation (figure 3A+B). However, liver haptoglobin mRNA and protein levels remained constant after balloon dilation (figure 3A+B). The maximum levels of arterial haptoglobin mRNA after balloon dilation were 345% compared to control livers whereas arterial haptoglobin protein increased to 209% of liver haptoglobin.

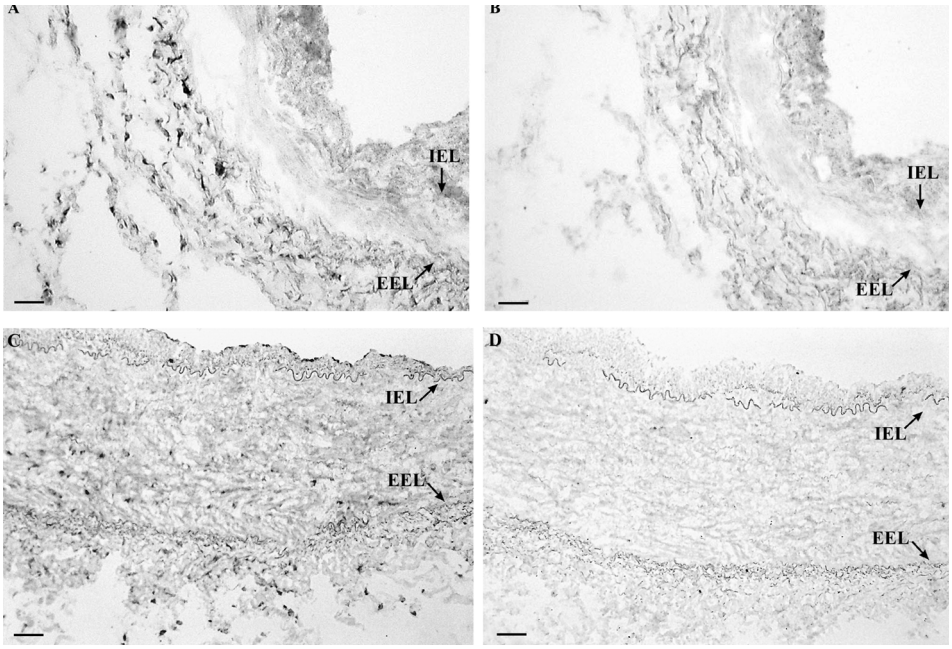

Figure 2. Localization of arterial haptoglobin mRNA
A) *in situ* hybridization on balloon dilated artery, 7 days after balloon dilation using an anti-sense probe for rabbit haptoglobin mRNA, and **B)** using a sense probe. **C)** *in situ* hybridization on human atherosclerotic coronary artery using an anti-sense probe for human haptoglobin mRNA, and **D)** using a sense probe. IEL= internal elastic lamina, EEL=external elastic lamina, magnification=100x, bar=50 µm

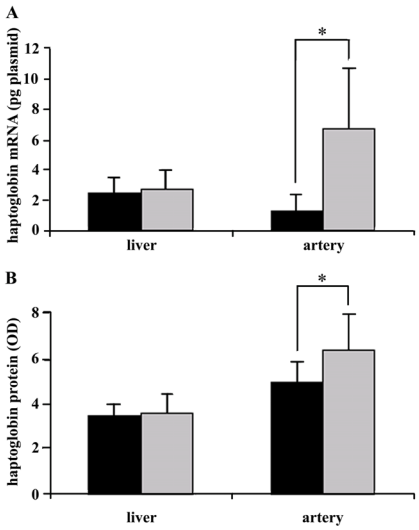

Figure 3. Tissue expression levels of haptoglobin, before and 3 days after balloon dilation
A) Haptoglobin mRNA expression in liver and artery. Haptoglobin mRNA is presented as the amount of plasmid containing the haptoglobin PCR product to which it correlates in the dilution series of this plasmid used in the quantitative real-time PCR. **B)** Haptoglobin protein expression in liver and artery. OD= optical density (arbritary units). n=4, * p=0.03, † p=0.02.

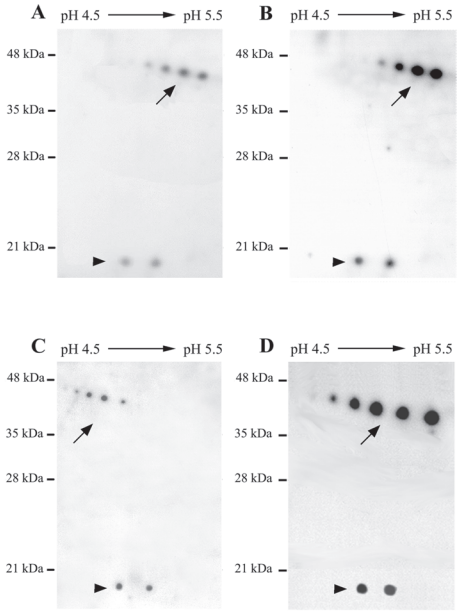

Figure 4. Differential expression of rabbit haptoglobin b-chains
Shown are western blots for haptoglobin protein after 2D-gel electrophoresis from **A)** control artery, **B)** balloon dilated artery, **C)** the liver and **D)** serum. The arrow head indicates haptoglobin α-chains, the arrow indicates haptoglobin β-chains.

Expression of arterial haptoglobin isoforms

To investigate variations in haptoglobin glycosylation, 2D gel electrophoresis and lectin affinity blotting was performed. Haptoglobin is a tetrameric glycoprotein consisting of two α- and two β-chains ($\alpha_2\beta_2$). After separation of total protein samples on 2D gel, a train of spots was detected representing different glycoforms of haptoglobin β-chain and two smaller spots representing the α-chains of haptoglobin. The 2D gels demonstrated that there were no differences in the number or position of arterial haptoglobin β-chains between control (figure 4A) and balloon dilated arteries (figure 4B) although total haptoglobin protein levels increased after balloon dilation. There were, however, large differences in localization of haptoglobin β-chains in arterial (figure 4A+B) and liver samples (figure 4C). Using the α-chains as a landmark on the gel, arterial haptoglobin β-chains were mainly located towards pH 4.5 while liver haptoglobin β-chains were located towards pH 5.5. In serum samples, both the liver and arterial haptoglobin β-chains

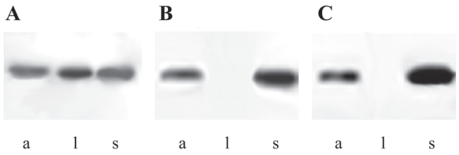

Figure 5. Tissue-specific glycosylation of haptoglobin
Shown is the presence of specific sugar groups on arterial haptoglobin using western blotting with haptoglobin antibodies **(A)** and the lectins GNA **(B)** and lotus tetragonolobus **(C)**. a=artery, l=liver, s=serum.

were detected (figure 4D).
As haptoglobin is a glycoprotein, lectin affinity blotting was used to determine differences in haptoglobin glycosylation between liver and arterial haptoglobin. The reactivity to the lectins GNA and lotus tetragonolobus demonstrated that terminal mannose and fucose residues were present on arterial haptoglobin but not on liver haptoglobin (figure 5). Serum samples also reacted positive for the two lectins.

Discussion

Recently, we demonstrated that haptoglobin is a novel cell migration factor and gelatinase-inhibitor that is expressed in arteries after sustained flow changes and involved in arterial restructuring[6]. Furthermore, haptoglobin has been associated with other extracellular matrix related processes like angiogenesis[13] and cancer[14]. Extracellular matrix turnover and arterial restructuring are important processes after balloon dilation. In the present study, we investigated the local expression of haptoglobin after balloon dilation and the presence of arterial haptoglobin glycoforms. Haptoglobin expression in balloon dilated rabbit arteries was increased within the first two weeks after balloon dilation with peak values at 7 days and correlated with the time period for increased MMP expression and cell migration after arterial injury[15-16]. Activation of MMP-2 has been associated with cell migration. There is, however, no linear correlation between active MMP-2 levels and cell migration. High levels of active MMP-2 result in excessive extracellular matrix and impaired cell migration while cells with intermediate active MMP-2 levels demonstrate efficient cell migration[17]. Thus, haptoglobin may provide cells with an additional regulation of MMP-2 activity[6], thereby providing the correct

substrate necessary for optimal cell migration.
Haptoglobin mRNA is only expressed by adventitial cells after balloon dilation and not by cells in the media or neointimal layer. These positive cells are probably fibroblasts, which are also the haptoglobin producing cells in the arterial wall after flow changes[6]. The adventitia is the first arterial layer that responds to arterial injury with enhanced extracellular matrix accumulation, cell proliferation[18] and MMP expression[19]. Moreover, adventitial fibroblasts migrate towards the subendothelial space, thereby contributing to neointima formation[20-21]. The adventitial expression of MMPs combined with elevated haptoglobin levels may facilitate the migration of adventitial fibroblasts.
Until recently, the liver was thought to be the major site of haptoglobin synthesis. However, haptoglobin is also expressed at basal levels in extrahepatic tissues and expression can be induced by lipopolysaccharide (LPS)[4-5]. Liver haptoglobin expression can be induced during systemic inflammation and liver injury which might explain the observed elevations in serum haptoglobin concentrations. However, until now it was unclear whether liver haptoglobin expression is also elevated after local stimulation of haptoglobin in extrahepatic tissues. In this study, we compared the relative levels of liver and arterial haptoglobin synthesis. Arterial haptoglobin expression increased early after balloon dilation at mRNA and protein levels. In contrast, liver haptoglobin mRNA and protein expression remained constant after balloon dilation. We infer that increased local arterial haptoglobin synthesis is responsible for the observed increases in arterial haptoglobin levels and that this is not due to extravasation of haptoglobin from serum.
Previously, an association had been found between altered glycosylation patterns of haptoglobin and the development and progression of various pathological processes[12]. Moreover, the type of glycosylation appeared to have prognostic values in certain pathological diseases[22-23]. However, the liver was always thought to be the production site of these abnormally glycosylated haptoglobin forms. In this study, we demonstrate that the rabbit artery produces an unique set of haptoglobin β-chains compared to the liver and that both forms can be detected in serum. This is in accordance with the human situation in liver and serum samples, as found in the SWISS 2D-PAGE database (http://www.expasy.ch/ch2d).
Lectin affinity blotting revealed that only arterial haptoglobin reacted to the lectins GNA and lotus tetragonolobus, confirming that differences in glycosylation are responsible for the specific arterial and liver haptoglobin glycoforms. This is in accordance with another study[24] showing that different haptoglobin β-chains react with different affinity to lectins. Serum samples also reacted positive with the used lectins, demonstrating that arterial haptoglobin is secreted into the serum. Since alterations in glycosylation are known to modify function, structure or targeting of proteins[25-27], it is conceivable that arterial haptoglobin has a different function compared to haptoglobin produced in the liver. However, the functional role of tissue-specific differences in haptoglobin glycosylation remains to be elucidated.
In summary, we found increased haptoglobin expression in the adventitia of rabbits after balloon dilation. Localization of arterial haptoglobin expression was confirmed in human atherosclerotic coronary arteries, supporting a role for haptoglobin in pathological arterial restructuring. Furthermore, we demonstrated that arteries produce a unique subset of haptoglobin β-chains compared to liver samples. Since human atherosclerotic arteries also express haptoglobin, this might point to a potential role for arterial haptoglobin glycoforms as a serum marker for atherosclerotic disease[28].

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V





Serum haptoglobin glycosylation patterns are not associated with atherosclerosis

Chapter VI

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Submitted

Serum haptoglobin glycosylation patterns are not associated with atherosclerosis.

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Background: Haptoglobin is an acute phase glycoprotein that is expressed in arteries and can be induced after blood flow changes or arterial injury. Arterial haptoglobin is differentially glycosylated compared to liver derived haptoglobin, allowing discrimination in serum samples. Previously, alterations in serum haptoglobin glycosylation have been associated with the progression and outcome of chronic inflammatory diseases. As atherosclerosis is an inflammatory disease, we investigated alterations in serum haptoglobin glycosylation patterns in atherosclerotic disease. Materials and methods: Serum was isolated from 80 atherosclerotic patients and 77 age, sex and risk factor matched control patients. All patients underwent non-invasive carotid artery duplex imaging to measure intima-media thickness (IMT). Total serum haptoglobin levels were measured using a sandwich ELISA. Lectin affinity blotting with four different lectins was used to investigate serum haptoglobin glycosylation patterns. Results: Total serum haptoglobin levels did not differ between atherosclerotic and control patients (p=0.63). In addition, no differences were found in serum haptoglobin glycosylation patterns (p>0.38). Neither total serum haptoglobin levels nor serum haptoglobin glycosylation patterns were associated with IMT in atherosclerotic or control patients (p>0.10). Conclusion: Serum haptoglobin glycosylation patterns do not differ in patients suffering from atherosclerotic disease indicating that it is unlikely to be a predictive serum marker for atherosclerotic disease.

Introduction

Atherosclerosis is being recognized as the major cause of death in Western society. Several studies have indicated that serum proteins can serve as markers for cardiovascular disease[1-2]. Haptoglobin is one of the major serum glycoproteins and levels are often increased during inflammation, tissue damage and diseases like cancer and arthritis[3]. Although haptoglobin is generally believed to be a liver-specific protein, it is also expressed at basal levels in various other extrahepatic tissues[4-5] including the arterial system[6]. Moreover, arterial haptoglobin expression is secreted into the serum and can be induced after sustained blood flow changes[6].

Studies investigating the feasibility of using serum haptoglobin levels as a marker for atherosclerosis revealed conflicting data. Snyder et al[7] found increased haptoglobin levels in patients with previous myocardial infarcts compared to healthy controls. This was supported by Lindberg et al[8] who described an association between serum haptoglobin levels and the prevalence of atherosclerotic disease. In contrast, Mori et al[1] found that serum haptoglobin levels were not associated with the severity of coronary atherosclerosis.

Atherosclerosis is considered to be an inflammatory disease[9]. Interestingly, other chronic inflammatory diseases like arthritis, Crohn's disease and ulcerative colitis are characterized by alterations in serum haptoglobin glycosylation patterns[10-12], even in the absence of changes in total serum haptoglobin levels. In addition, serum haptoglobin glycosylation patterns can vary between different diseases[10, 13] and appear to be

predictive for the progression and outcome of inflammatory diseases[10, 14]. Previously, we demonstrated in rabbits that arterial haptoglobin has a unique glycosylation pattern compared to liver haptoglobin and that these arterial haptoglobin glycoforms can be detected in the serum (unpublished results). However, it is not known whether glycosylation patterns of total serum haptoglobin will change during atherosclerotic disease.

Based on the tissue-specific glycosylation of haptoglobin and previous studies on alterations in serum haptoglobin glycosylation in other inflammatory diseases, we hypothesized that serum haptoglobin glycosylation patterns might alter in atherosclerotic disease. In addition, total serum haptoglobin levels and haptoglobin glycosylation patterns were associated with carotid artery intima-media thickness, a surrogate marker for the extent of atherosclerotic disease.

Material and methods

Patient population

All patients were participants of the Second Manifestations of ARTerial disease (SMART) study, an ongoing, single-center, prospective cohort study of approximately 3500 patients referred to the University Medical Center Utrecht for the first time because of atherosclerotic vascular disease or for treatment of atherosclerotic risk factors.

From the SMART study database, 80 atherosclerotic patients with a history of atherosclerotic vascular disease, atherosclerotic disease at the time of hospital-entry and one cardiovascular risk factor were selected. In addition, these patients were also screened for the presence of clinically silent atherosclerotic lesions in the carotid and femoral arteries and aorta. Atherosclerotic luminal narrowing in the femoral artery was evaluated by measuring the ratio of the systolic blood pressure measured at the ankle to the systolic blood pressure measured in both arms (ankle-brachial pressure index, ABPI). When patients had a lowered ABPI (<0.9), they were classified having silent atherosclerosis. Plaques and stenosis of the common and internal carotid arteries on both sides were measured with Doppler-assisted Duplex scanning. When the carotid stenosis exceeded the 70%, patients were diagnosed having clinically silent atherosclerosis. Furthermore, ultrasonography of the abdomen was performed to measure the anteroposterior juxtarenal diameter and the distal anteroposterior diameter of the aorta. As a control group, 77 age-, sex- and risk factor matched patients not having clinically evident or silent atherosclerosis were selected.

Patient sera

Blood samples were collected from each individual and serum was isolated. Samples were stored in liquid nitrogen until analysis.

Intima media thickness measurements

In all patients, intima-media thickness (IMT) was measured in the left and right common carotid arteries. The left and right common carotid arteries were examined in anterolateral, posterolateral and mediolateral direction using a ATL Ultramark 9 (Advanced Technology Laboratories) equipped with a 10 MHz linear array transducer. The total intima-media surface of the selected area was calculated on-line using built-in software of the ultrasound system. The mean IMT of the six measurements in each patient was calculated. Patients were divided in quartiles on the basis of IMT.

Haptoglobin enzyme-linked immunosorbent assay (ELISA)

In microtiter plates, wells were coated $^{o}/_{n}$ at room temperature (RT) with 1 ng/ml goat-α-human haptoglobin (DAKO). After coating, the wells were washed three times with PBST (PBS-0.1% Tween-20) and incubated for 30 minutes with 1% block solution (PBST+1% non-fat dry milk). Wells were incubated for 1 hour at 37°C with patient serum (1:500.000 dilution in 1% block solution), followed by three washes with PBST. Subsequently, the wells were incubated for 1 hour at 37°C with goat-α-human haptoglobin (1:4000, Sigma) and rabbit-α-goat peroxidase (1:10.000, Sigma). After three washes with PBST, ortho-phenylene diamine/H_2O_2 substrate solution was added. The colorimetric reaction was stopped by adding 4M H_2SO_4 and the optical density was measured at 490/650 nm. Serum haptoglobin concentrations were calculated using a standard curve containing four reference concentrations of purified human serum haptoglobin (Sigma).

Haptoglobin glycosylation: lectin affinity blotting

Serum samples (0.1 µl/lane) were separated under reducing conditions on a 10% sodium dodecyl sulfate polyacrylamide gel. Proteins were electrophoretically transferred in blotting buffer (3.03 g/L Tris 14.4 g/L Glycine, 20% methanol) onto Hybond ECL (Amersham). Incubation with biotinylated lectins GNA, SNA, WGA and lotus tetragonolobus (1:1000, EY Laboratories) occurred in 5% block solution (PBST+5% non-fat dry milk) for 1 hour at RT followed by three washes with PBST. Incubation was continued with streptavidin peroxidase (1:1000, SBA) for 1 hour at RT in 5% block solution, followed by three washes with PBST and a single wash with PBS. Detection occurred using the enhanced chemiluminescence kit (NEN Life Science products) and exposure to X-Omat Blue XB-1 films (Kodak).

Statistics

Data are presented as mean±standard error of the mean (sem). The independent *t*-test was used to compare total serum haptoglobin levels and serum haptoglobin glycosylation between control and atherosclerotic patients. Linear regression analysis was performed to investigate the relation between mean IMT and serum haptoglobin levels and glycosylation patterns in the two patient groups. One-way ANOVA was performed to investigate differences in serum haptoglobin levels and glycosylation patterns between the quartiles of IMT. P values <0.05 were considered to be statistically significant.

Table I. Patient characteristics of the study groups

	control	atherosclerosis
no. of subjects (male)	77 (38)	80 (40)
age, years	56.8 ± 9.4	59.7 ± 10.8
BMI, kg/mm^2	26.1 ± 4.2	26.1 ± 3.3
smoking, no. of subjects	51	72
(a)symptomatic carotid stenosis	0	39
peripheral arterial disease	0	31
abdominal aortic aneurysm	0	6
hypertension, no. of subjects	5	5
diabetes, no. of subjects	7	7
hyperlipidemia, no. of subjects	65	68
systolic blood pressure, mm Hg	134 ± 16	142 ± 17
diastolic blood pressure, mm Hg	80 ± 9	76 ± 9
mean IMT, mm	0.8 ± 0.2	0.9 ± 0.3
ABPI <0.9, no. of subjects	0	35
cholesterol, mmol/L	6.8 ± 2.1	5.7 ± 1.2
HDL cholesterol, mmol/L	1.1 ± 0.4	1.1 ± 0.3
triglycerides, mmol/L	3.1 ± 3.0	2.0 ± 0.9

Results

Patient characteristics

The patient characteristics are summarized in table I. Atherosclerotic and control patients were matched for age, sex and risk factors. Since there were no differences in serum haptoglobin levels and glycosylation patterns between males and females, the data from both sexes were pooled.

Serum haptoglobin levels and glycosylation patterns

Serum haptoglobin levels ranged from 0.01 to 7.80 g/L. There were no differences in serum haptoglobin levels between patients with atherosclerosis and control patients (p=0.63) (figure 1). Overall, serum haptoglobin levels were within the normal range between 0.4 to 3.8 g/L[16] (mean 1.8±0.2 g/L). Using a cut-off point of serum haptoglobin levels <0.4 g/L, four atherosclerotic patients and three control patients revealed low but detectable levels of serum haptoglobin and could be classified as hypo-haptoglobinemic.

Lectin affinity blotting was used to measure haptoglobin glycosylation as lectins recognize specific carbohydrates that are present on glycoproteins (figure 2A). Analysis of serum haptoglobin glycosylation revealed no differences in haptoglobin glycosylation patterns between atherosclerotic and control patients for all four tested lectins (for all groups p>0.38) (figure 2B), even after adjustment for total serum haptoglobin levels.

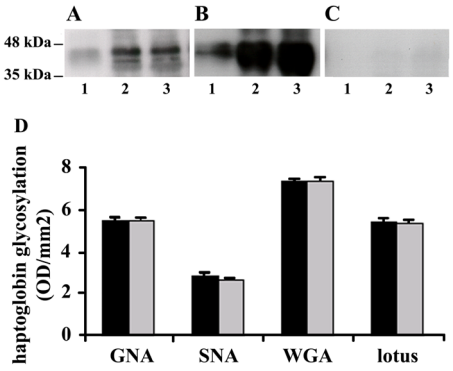

Figure 2. Glycosylation patterns of serum haptoglobin in atherosclerotic and control patients

A) Lectin-affinity blot using lectin GNA (representative for the other lectins used in this study). **B)** Western blot using a goat-α-human haptoglobin antibody. **C)** Western blot using a goat-α-human haptoglobin antibody. Blocking the goat-α-human haptoglobin antibody with 4 µg purified human serum haptoglobin resulted in the disappearance of haptoglobin bands. Lane 1= purified human haptoglobin, lane 2= atherosclerotic patient, lane 3= control patient. **D)** Glycosylation patterns of serum haptoglobin from control (black bars) and atherosclerotic (grey bars) patients.

Relation between haptoglobin and IMT

Linear regression analysis did not reveal any correlation between total serum haptoglobin levels and IMT in atherosclerotic and control patients (p>0.10) (data not shown). In addition, no association was found between serum haptoglobin glycosylation and IMT (for all groups p>0.17). Subdivision of IMT into quartiles neither revealed a relation between IMT and total serum haptoglobin levels nor between IMT and glycosylation patterns (table II).

Table II. Association between common carotid intima-media thickness (IMT) and total serum haptoglobin levels or glycosylation patterns

P values <0.05 are considered to be statistically significant

	IMT (quartiles)				ANOVA
	1e	2e	3e	4e	P
haptoglobin	2.0±0.3	1.9±0.3	1.8±0.3	1.9±0.3	0.90
GNA	5.8±0.2	5.3±0.2	5.5±0.2	5.4±0.2	0.33
SNA	2.7±0.2	2.8±0.1	2.7±0.1	2.8±0.2	0.88
WGA	7.2±0.2	7.3±0.2	7.7±0.4	7.2±0.3	0.55
lotus	5.0±0.3	5.3±0.3	5.2±0.3	5.7±0.3	0.58

Discussion

In the present study, we measured total serum haptoglobin levels and glycosylation patterns in 80 atherosclerotic patients and 77 age, sex and risk factor matched controls. However, no association was found between total serum haptoglobin levels and atherosclerotic disease or IMT in the carotid artery, which is a surrogate measure for the extent of atherosclerosis. In addition, there were no differences in serum haptoglobin glycosylation patterns between atherosclerotic and control patients. Serum haptoglobin glycosylation patterns were not related to mean IMT.

Based on previous studies, we hypothesized that an association between serum haptoglobin glycosylation patterns and atherosclerotic disease might be present. Our hypothesis was based on three observations. First, haptoglobin is expressed in arteries and can be induced after sustained blood flow changes[6]. Secondly, arterial

haptoglobin is differentially glycosylated compared to liver-derived haptoglobin and these arterial haptoglobin glycoforms can be detected in the serum (unpublished results). Finally, several sero-epidemiological studies have demonstrated that haptoglobin glycosylation patterns change during chronic inflammatory diseases[10-11, 13], suggesting that serum haptoglobin glycosylation patterns reflect the presence of active inflammatory disease.

Although there seems to be a relation between serum haptoglobin glycosylation patterns and the outcome of inflammatory diseases[10-11, 13], no relation in time is described for haptoglobin glycosylation and the inflammatory status of patients. Fucosylation of haptoglobin is specifically increased in patients with active arthritis and not in patients with inactive arthritis[16]. No association has been described between haptoglobin glycosylation and disease activity in patients with Crohn's disease[17]. Thus, activity of disease might influence temporal glycosylation patterns of haptoglobin. The patient population that was selected for the present study all entered the hospital for a current manifestation of atherosclerotic disease suggesting that the negative results cannot be explained by an inactive phase of atherosclerotic disease.

In the present study, we investigated four different serum haptoglobin glycosylation patterns with relationship to with atherosclerotic disease. Alterations in sialylation, glucosaminylation and fucosylation of serum haptoglobin (respectively lectins SNA, WGA and lotus tetragonolobus) have previously been described to be associated with inflammatory diseases[10, 13-14, 16, 18]. In addition, differences in mannosylation (lectin GNA) have been used to distinguish arterial haptoglobin from liver-derived haptoglobin in rabbits (unpublished results). Although we did not observe an association between haptoglobin glycosylation and atherosclerotic disease, it is still possible that other serum haptoglobin glycosylation patterns are associated with atherosclerotic disease[13].

Atherosclerosis involves a chronic inflammatory process that takes several years and gradually progresses from neointima formation to advanced atherosclerotic lesions. This slow development of atherosclerotic lesions may not be sufficient to induce arterial haptoglobin expression or only at very low levels. Although haptoglobin expression has been demonstrated in atherosclerotic arteries, low arterial expression levels will make it difficult to detect changes in the subpopulation of arterial-derived haptoglobin in serum due to the high baseline levels of haptoglobin in serum (0.4-2.6% of total plasma proteins). In addition, arterial haptoglobin expression may only be induced after severe events like plaque rupture, which only occurs in small parts of the arterial system.

Animal models have demonstrated that arterial haptoglobin expression is increased early after flow changes[6] and returns to baseline levels within 2-3 weeks after induction. Arterial restructuring, in contrast, continues to progress up to 4 weeks after balloon angioplasty, implying that haptoglobin is only important during the initiation of arterial restructuring. In general, patients with atherosclerosis enter the hospital years after the initiation of atherosclerosis as the progression towards severe atherosclerotic plaques takes several years. In this study, we compared patients with progressed atherosclerosis to healthy control patients. However, it might be interesting to investigate whether total serum haptoglobin levels or haptoglobin glycosylation patterns in patients early after balloon angioplasty or stenting are predictive for the occurrence of (in-stent) restenosis.

The absence of a relation between total serum haptoglobin levels and atherosclerosis and an equal distribution of hypohaptoglobinemic people between the control and atherosclerotic patient groups suggests that haptoglobin is not involved in arterial restructuring during atherosclerosis. However, several studies have indicated that haptoglobin does play an important role during arterial restructuring. Although haptoglobin knockout mice are viable and demonstrate no obvious symptoms[19], these mice have impaired arterial restructuring after blood flow changes[6]. In addition, haptoglobin polymorphisms in humans have been associated with various cardiovascular diseases including a higher prevalence of coronary artery disease[20] and more restenosis after balloon angioplasty[21] or sten-

ting[22]. These studies indicate that haptoglobin may not be essential for normal vessel wall homeostasis but does play an important role after arterial injury and the subsequent repair process. In summary, we have demonstrated that total serum haptoglobin levels and haptoglobin glycosylation patterns are not associated with atherosclerosis. Hence, serum haptoglobin glycosylation patterns are unlikely to be a predictive serum marker for atherosclerotic disease.

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General discussion

Chapter VII

General discussion

The arterial wall consists of various cell types that are embedded in extracellular matrix. Together, cells and extracellular matrix maintain the structural integrity of the artery. In response to injury, the artery starts a complex but controlled healing response including processes like cell-cell and cell-matrix interactions, cell migration and remodeling of the extracellular matrix.

Collagen is the main component of the extracellular matrix in arteries and plays an important role during arterial restructuring. Besides providing the artery with structural strength[1], the composition of collagen fibers is also vital for controlling many cellular processes like cell phenotype, protein synthesis and cell migration[2-3]. This is, however, a complex system that requires tight control of collagen turnover. Intact collagen fibers need to be partially degraded for cell detachment and cell migration to occur[4]. However, total degradation of collagen around the cell would result in complete cell detachment and subsequent cell death[5]. In addition, active collagen synthesis is essential for cell migration[6], probably by providing cells the correct substrate to form a temporary matrix that is suitable for cell migration.

As collagen turnover is important for arterial restructuring, many studies have investigated the various pathways involved in collagen synthesis and degradation[7-8]. Although some studies investigated the effect of altered matrix composition on cell behavior and signaling[9-12], most studies have focussed on matrix turnover itself and the proteins involved in matrix synthesis and degradation[13-15]. There are, however, still many proteins that play a role during arterial restructuring that have not been identified yet. One of the recently identified proteins that is involved in arterial restructuring is haptoglobin[16]. Haptoglobin is expressed at basal levels in the arterial wall and arterial expression can be induced after sustained flow changes[16] or balloon dilation (see chapter 2 and 5). Haptoglobin can function as a gelatinase-inhibitor and haptoglobin deficiency results in diminished cell migration. Supplementation of exogenous haptoglobin to haptoglobin knockout cells restores their capacity to migrate[16]. In addition, haptoglobin knockout mice demonstrated delayed arterial restructuring after sustained flow changes[16], suggesting an important role for haptoglobin during arterial repair.

Haptoglobin function

Haptoglobin is an acute phase serum protein that comprises up to 2.6% of the total protein in serum. Haptoglobin is mostly recognized for its high binding affinity to hemoglobin[17] and thought to be important for the clearance of free hemoglobin from the serum. However, clearance of free hemoglobin from the serum and subsequent liver degradation is normal in haptoglobin knockout mice[18], indicating that haptoglobin is not essential for the clearance of free hemoglobin. This is in accordance with previous studies demonstrating that isolated liver cells take up free hemoglobin in the absence of haptoglobin[19] and that free hemoglobin is cleared at a faster rate from the serum than hemoglobin-haptoglobin complexes[20].

Recently, several studies have demonstrated various other biological functions for haptoglobin of which some have been related to the high binding affinity of haptoglobin to hemoglobin. One of the best described functions of haptoglobin that is related to hemoglobin binding is its role as antioxidant. The hemoglobin-haptoglobin complexes reduce the formation of free radicals like superoxide and hydroxyl that are catalyzed by heme iron[21]. Heme iron also catalyzes the oxidation of lipoproteins which can cause damage to cells and tissue[22]. Thus, as a consequence of the capture of free hemoglobin by haptoglobin, heme iron is unavailable for iron-driven oxidation reactions.

The inhibitory effect of haptoglobin on some enzymes like prostaglandin synthase also appear to depend on the availability of heme-derived iron. Binding of free heme controls the peroxidase activity of prostaglandin synthase, an enzyme that is inhibited by haptoglobin[23-24]. However, the observed inhibition of gelatinase activity by haptoglobin[16] is presumably not related to the availability of free iron. MMP activity assays have demonstrated inhibition of gelatinase activity

by haptoglobin in the absence of heme-derived iron. In contrast, cell culture experiments demonstrated that supplementation of iron to cells results in increased MMP-2 activity[25].

Another function of haptoglobin involves the modulation of the immune response. Haptoglobin expression is induced during inflammation and has been described to act as a potent immunosuppressor. Haptoglobin can function as a natural antagonist for receptor-ligand activation of the immune system[26] and inhibits lectin- or LPS-induced lymphocyte transformation[27]. Furthermore, haptoglobin binds to several cell types of the immune system and may influence their function. A specific interaction between haptoglobin and CD22[28] has been described, which is a B-cell receptor that mediates B-cell interactions with other cell types. Haptoglobin can also interact with the Mac-1 (CD11b/CD18) receptor[29] that belongs to the integrin family and is involved in cell-cell and cell-matrix interactions. Through interaction with this receptor, haptoglobin might influence the Th1/Th2 balance towards a dominant Th1 response[30]. In addition, haptoglobin can exert an anti-inflammatory effect by inhibition of inflammatory mediators like prostaglandin synthase[31] and cathepsins[32].

Haptoglobin and tissue repair

Studies investigating the role of haptoglobin in hemoglobin clearance demonstrated that haptoglobin knockout mice were more susceptible to tissue damage and showed delayed tissue repair[18] then wild type mice. The possible involvement of haptoglobin in tissue repair is further supported by the observation that haptoglobin knockout mice have delayed arterial repair after ligation of the carotid artery[16].

The exact mechanism by which haptoglobin modulates tissue damage and the repair process is still not known but there are several possible explanations, based on the various functions that have been described for haptoglobin. First, haptoglobin may function as an anti-oxidant through it's high affinity binding capacity to hemoglobin. The increased accumulation of free hemoglobin in the kidney of haptoglobin knockout mice during hemolysis results in increased lipid peroxidation and eventually in tissue injury and delayed repair[18]. However, there is no evidence for increased hemoglobin accumulation in the carotid artery after ligation[16]. Secondly, haptoglobin may influence the tissue healing response by modulating cell migration. Cell migration is an important process during the healing response[33] and haptoglobin has been identified as a natural gelatinase-inhibitor and an important factor during cell migration[16]. Supplementation of exogenous haptoglobin to haptoglobin knockout cells restored their capability to migrate. In addition, haptoglobin stimulates angiogenesis[34] which also depends on cell migration.

Haptoglobin and cell migration

The involvement of haptoglobin, as an endogenous gelatinase inhibitor, in cell migration seems paradoxical as many studies have linked cell migration and cell invasion to increased MMP activity. It is generally believed that degradation of pre-existing collagen fibers by MMPs is necessary for cell migration to occur. However, uncontrolled degradation could impede this process, since extracellular matrix components are required for cell migration and cell survival.

Nowadays, there is evidence that only limited degradation of the extracellular matrix is sufficient to promote cell migration and subsequent matrix remodeling. Degradation of a gelatin matrix by migrating cells is not random or total but involves the reorganization of extracellular matrix[35]. Furthermore, contraction of the extracellular matrix requires only subtle rearrangements of extracellular matrix [36-37]. Apparently, MMPs do not randomly degrade extracellular matrix but are involved in a highly localized, tightly controlled and efficient reorganization of the extracellular matrix to provide the correct substrate necessary for cell migration or matrix remodeling. Moreover, there is increasing evidence that soluble MMPs are less active than cell-surface associated MMPs[38], suggesting that MMP-mediated degradation of the extracellular matrix is restricted to the immediate surrounding of cells[39-40].

Several studies have demonstrated that non-selective MMP inhibitors, either synthetic compounds[41-42] or the endogenously expressed TIMPs[43-44], can inhibit cell migration. However,

these studies did not focus on the role of individual MMPs, making it difficult to say whether collagenases, gelatinases or a combina-tion of both classes of MMPs are necessary for cell migration. Studies investigating the role of individual MMPs in cell migration after arterial injury are descriptive and only demonstrate a relation in expression patterns over time between increased MMP activity and cell migration but not a functional relationship. In addition, studies using MMP knockout mice failed to demonstrate an essential role for individual MMPs in cell migration. Most MMP knockout mice lack a lethal embryonic phenotype and deliver healthy offspring with limited morphologic abnorma-lities[45-49]. The only exception is the deletion of MT1-MMP which causes dwarfism, arthritis and connective tissue diseases related to impaired collagen degradation[50]. Apparently, multiple back-up mechanisms exist that can compensate for the loss of a single MMP.

The exact mechanism by which MMPs regulate cell migration remains to be elucidates. As haptoglobin can inhibit gelatinase activity, it may provide the necessary fine-tuning of gelatinase activity that is required during the tightly controlled process of cell migration. Inhibition of gelatinase activity by haptoglobin will result in the accumulation of gelatin around the cell that can serve as a temporary matrix for cell migration (figure 1). In contrast, nonselective MMP inhibitors inhibit more than one class of MMPs or may exert their effect by inhibiting MMPs that are more upstream in the MMP cascade like the collagenases. This may result in complete inhibition of gelatin formation and thus inhibition of cell migration due to the absence of the correct substrate.

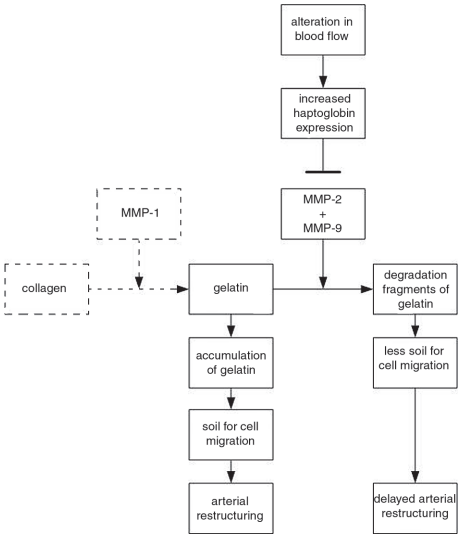

Figure 1. Schematic diagram of the hypothesized gelatinase-inhibition of haptoglobin.
Inhibition of the gelatinases MMP-2 and MMP-9 by haptoglobin results in improved cell migration and arterial restructuring. Dashed lines indicate proteins and pathways involved in the formation of gelatin.

Haptoglobin glycosylation

Haptoglobin is a glycoprotein with 4 *N*-linked glycosylation sites on the β-chain. Previously, sero-epidemiological studies have demonstrated alterations in serum haptoglobin glycosylation that have been associated with disease development and progression[51]. Haptoglobin is highly fucosylated in ovarian cancer[52] and inflammatory joint diseases[53] whereas patients with Crohn's disease have increased sialylated serum haptoglobin levels[54]. As haptoglobin was thought to be produced mainly in the liver and secreted into the serum, it was always assumed that the abnormally glycosylated haptoglobin was produced by the liver. However, recent studies have demonstrated that haptoglobin is also locally expressed at extrahepatic sites[16, 55] and that local haptoglobin expression is increased during pathological processes (see chapter 4) or inflammation[56], suggesting that extrahepatic haptoglobin production can also be responsible for the alterations in serum haptoglobin glycosylation patterns.

Two-dimensional gel analysis and lectin affinity blotting demonstrated that extrahepatic tissues can produce distinct haptoglobin glycoforms compared to liver-derived haptoglobin (see chapter 5). In addition, these artery-derived haptoglobin glycoforms can be detected in the serum. As atherosclerotic arteries produce haptoglobin, this implies that serum haptoglobin glycosylation patterns may be used as a marker for cardiovascular disease. Unfortunately, we observed no alterations in total serum haptoglobin levels or serum haptoglobin glycosylation

patterns between atherosclerotic and control patients (see chapter 6). This may be related to no or only small increases in haptoglobin expression in atherosclerotic arteries. As haptoglobin is one of the major serum proteins and present at relative high concentrations in the serum, this makes it difficult to detect small differences in serum haptoglobin levels.

Although the general role of protein glycosylation has not been completely established, recent studies have indicated that posttranslational glycosylations may exert multiple effects. Besides modulation of protein solubility[57], protein folding[58], cell surface expression[59-60] and stabilization of protein conformation[61], glycosylation can also modulate biological functions of proteins. Glycosylation of MD-2 and Toll-like receptor 4 is essential for binding of LPS to MD-2/TLR-4 complexes and cell activation[62]. Ligand-binding[63] as well as ligand-induced receptor internalization[64] is modulated by glycosylation. Furthermore, glycosylation can influence enzyme activity by increasing enzyme stability[65] and modifying of catalytic activity[66-67].

The finding that arteries produce a unique set of haptoglobin glycoforms suggests that local haptoglobin expression provides the tissue with a source of functionally different haptoglobin compared to serum haptoglobin. However, no information is available about the function of the glycosylation sites in haptoglobin and it remains to be elucidated whether alterations in haptoglobin glycosylation can modulate haptoglobin function.

As haptoglobin phenotype in humans is determined by the haptoglobin α-chain whereas glycosylation occurs on the haptoglobin β-chain, it is not likely that glycosylation will directly influence the observed functional differences between the haptoglobin phenotypes. However, it is possible that formation of multimeric haptoglobin complexes is influenced by the presence or absence of specific sugar groups on the haptoglobin β-chain which may stochiometrically inter-fere with haptoglobin function.

Haptoglobin in cardiovascular disease

People with haptoglobin deficiency (anhaptoglobinemia) as well as haptoglobin knockout mice are viable and demonstrate no higher risk to cardiovascular disease during normal live. However, haptoglobin knockout mice show delayed arterial restructuring after sustained flow changes. In addition, haptoglobin phenotype has been associated with the development of coronary artery disease[68] and restenosis after balloon angioplasty[69] or stenting[70]. Therefore, we infer that artery-derived haptoglobin is not essential during normal vessel wall homeostasis but may play an important role in pathological arterial processes like atherosclerosis and arterial restructuring after balloon angioplasty.

Although haptoglobin is present in the serum of all mammals, only humans have a polymorphism with three phenotypes due to the presence of a haptoglobin α2-chain. Interestingly, the three main phenotypes of haptoglobin (Hp1-1, Hp2-1 and Hp2-2) are characterized by differences in biophysical and biochemical properties. A possible explanation for the involvement of haptoglobin phenotype in cardiovascular disease is oxidative stress which has been implicated in the initiation and pathogenesis of atherosclerosis[71]. The three phenotypes of haptoglobin have small differences in their antioxidant capacity with Hp1-1 providing the best protection against oxidative stress due to free hemoglobin[72]. The relative small differences in antioxidant capacity in vitro may be dramatically amplified in vivo due to the size of haptoglobin multimers. Smaller haptoglobin multimers like monomeric haptoglobin 1-1 have better sieving capacities to get into extravascular fluids or tissue and can therefore better function as an antioxidant. In addition, phenotypic differences for haptoglobin have been described in modulation of the immune response[73] and for the angiogenic properties of haptoglobin[34]. Hence, haptoglobin phenotype may influence the development and progression of cardiovas-cular disease through several mechanisms instead of just one.

Concluding remarks and future aspects

Collagen turnover is an important process during arterial restructuring. Using a subtraction PCR to identify new proteins involved in collagen turnover, we demonstrated increased arterial

haptoglobin expression after sustained flow changes or arterial injury. Haptoglobin was found to be a gelatinase-inhibitor and involved in cell migration and arterial restructuring. In addition, arteries produce specific haptoglobin b-chain glycoforms that may imply functional differences between liver- and arterial-derived haptoglobin. We have previously demonstrated that arteries produce specific haptoglobin glycoforms compared to liver-derived haptoglobin. Although it is known for other proteins that changes in glycosylation can alter protein function, we did not demonstrate that alterations in haptoglobin glycosylation modulate haptoglobin function. Therefore, it would be interesting to study arterial haptoglobin glycosylation in more depth and investigate whether differential glycosylation of arterial haptoglobin indeed modulates the function of haptoglobin.

Although we did not observe a relation between total haptoglobin serum levels and serum haptoglobin glycosylation patterns, we did not exclude the possibility that haptoglobin can function as a serum marker for acute arterial restructuring after balloon angioplasty or stenting. As arterial haptoglobin is involved in arterial restructuring, changes in total serum haptoglobin levels or haptoglobin glycosylation patterns in patients early after balloon angioplasty or stenting may be predictive for the occurrence of (in-stent) restenosis.

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Summary

Summary

Several studies have demonstrated that collagen turnover is important during arterial restructuring. However, the precise mediators involved in collagen turnover are still not known. In order to develop specific inhibitors to prevent arterial restructuring, it is essential to get a better understanding of the molecular pathways involved in collagen turnover. We used a subtraction PCR to identify new candidate proteins that are involved in arterial restructuring and to get a better insight in these pathways. One of the identified proteins is the acute phase glycoprotein haptoglobin. In this thesis, we investigated the arterial expression, regulation and function of haptoglobin.

Chapter 1 is a general introduction that provides an overview about the role of collagen turnover in arterial restructuring after balloon angioplasty. In addition, background information is given about haptoglobin.

Although many studies have demonstrated an essential role for collagen turnover during arterial restructuring, there are still unidentified proteins that are involved in collagen turnover. In **chapter 2**, subtraction PCR is used as an open approach to identify novel, differentially expressed genes involved in collagen turnover and cell migration during arterial restructuring. Haptoglobin was one of the identified genes and found to be expressed by adventitial fibroblasts, early after flow changes. In vitro assays demonstrated that haptoglobin is a gelatinase inhibitor and important for cell migration. Furthermore, arterial restructuring after flow changes was delayed in haptoglobin knockout mice.

Previous studies have mainly investigated the regulation of haptoglobin expression in the liver. The study described in **chapter 3** was designed to identify the mediators involved in arterial haptoglobin expression after flow changes. Inhibition of NO synthesis resulted in decreased arterial haptoglobin expression after flow changes, coinciding with decreased arterial IL-6 expression. IL-6 knockout mice were used to confirm a regulatory role of IL-6 in arterial haptoglobin expression. As haptoglobin expression was normal in the IL-6 knockout mice, this points to backup mechanisms for the regulation of arterial haptoglobin expression.

Cell migration and remodeling of the extra-cellular matrix are important during various physiological and pathological processes. As haptoglobin plays an important role in cell migration and remodeling of the extracellular matrix, we investigated in **chapter 4** the local expression of haptoglobin in two pathological tissues that are characterized by these two processes. Previously, sero-epidemiological studies have demonstrated that arthritis and cancer are characterized by increased serum haptoglobin levels. In this study, we demonstrated that local haptoglobin expression is also locally increased in arthritic and cancer tissue.

Haptoglobin is thought to be produced mainly in the liver. However, several studies have also described extrahepatic haptoglobin expression. As haptoglobin is present at high concentrations in the serum, the function of local haptoglobin expression is not completely understood. In **chapter 5**, we investigated whether alterations in haptoglobin glycosylation could explain the necessity of local haptoglobin expression in the arterial wall. Haptoglobin expression and glycosylation was studied in balloon dilated rabbit arteries. Balloon dilation increased arterial haptoglobin expression within the first two weeks after injury whereas liver haptoglobin expression remained constant. There were no differences in arterial haptoglobin glycosylation after balloon dilation. However, arterial haptoglobin was differentially glycosylated compared to liver haptoglobin and these arterial haptoglobin glycoforms could be detected in the serum.

Previously, alterations in serum haptoglobin glycosylation have been associated with the progression and outcome of different diseases. In **chapter 6**, we investigated alterations in serum haptoglobin glycosylation patterns in atherosclerotic patients. There were no significant differences in total serum haptoglobin levels or serum haptoglobin glycosylation patterns between patients with atherosclerosis and patients with only risk factors. In addition, no relation was found between total serum haptoglobin levels and glycosylation patterns with intima-media thickness in the carotid artery, which is a measure for the extent of atherosclero-sis. These

results indicate that serum haptoglobin glycosylation patterns are not suitable to use as a serum marker for atherosclerotic disease.
Chapter 7 is a general discussion of the results from the aforementioned studies.





Samenvatting

Samenvatting

Meerdere studies hebben aangetoond dat de omzetting van collageen belangrijk is tijdens herstructurering van de vaatwand. Het is echter nog steeds niet bekend welke regulatoren precies een rol spelen in de omzetting van collageen. Voor de ontwikkelen van specifieke remmers om herstructurering van de vaatwand te voorkomen is een beter begrip nodig van alle moleculaire processen die betrokken zijn bij de omzetting van collageen. We hebben gebruik gemaakt van een subtractie PCR om nieuwe kandidaat eiwitten te identificeren die betrokken zijn bij de herstructurering van de vaatwand en om een beter begrip te krijgen in de processen die hierbij van belang zijn. Een van de eiwitten die is opgepikt m.b.v. de subtractie PCR is het acuut fase eiwit haptoglobine. In dit proefschrift beschrijven we de arteriële expressie, regulatie en functie van haptoglobine.

Hoofdstuk 1 is een algemene inleiding die een overzicht geeft over de rol van de omzetting van collageen tijdens herstructurering van de vaatwand na dotteren. Daarnaast wordt achtergrondinformatie gegeven over haptoglobine.

Hoewel veel studies hebben aangetoond dat omzetting van collageen belangrijk is voor herstructurering van de vaatwand, zijn er nog steeds niet geïdentificeerde eiwitten die betrokken zijn bij de omzetting van collageen. In **hoofdstuk 2** wordt een subtractie PCR gebruikt om nieuwe eiwitten te ontdekken die verhoogd of verlaagd tot expressie komen en betrokken zijn bij de omzetting van collageen en cel migratie tijdens herstructurering van de vaatwand. Een van de opgepikte eiwitten is haptoglobine dat vlak na veranderingen in de bloedstroom wordt aangemaakt door fibroblasten in de adventitia. In vitro studies toonden aan dat haptoglobine gelatinases kan remmen en een belangrijke rol speelt tijdens cel migratie. Daarnaast werd aangetoond dat herstructurering van de vaatwand vertraagd is in haptoglobine knockout muizen.

Eerdere studies hebben voornamelijk de regulatie van haptoglobine synthese bestudeerd in de lever. De studie die beschreven wordt in **hoofdstuk 3** was opgezet om regulatoren te identificeren die betrokken zijn in de expressie van haptoglobine in de vaatwand na veranderingen in de bloedstroom. Remming van NO synthese leidde tot verlaagde haptoglobine expressie na verandering in bloedstroom, tegelijkertijd met verlaagde aanmaak van IL-6 in de vaatwand. IL-6 knockout muizen werden gebruikt om de regulerende rol van IL-6 in haptoglobine expressie te bevestigen. De expressie van haptoglobine was echter normaal in IL-6 knockout muizen, duidend op backup mechanisme voor de regulatie van haptoglobine expressie.

Cel migratie en remodeleren van de extracellulaire matrix zijn belangrijke tijdens meerdere fysiologische en pathologische processen. Omdat haptoglobine een belangrijke rol speelt tijdens cel migratie en remodeleren van de extracellulaire matrix hebben we in **hoofdstuk 4** de locale productie van haptoglobine onderzocht in twee pathologische weefsels die gekenmerkt worden door deze twee processen. Eerder hebben seroepidemiologische studies al aangetoond dat artritis and kanker gekenmerkt worden door verhoogde concentraties haptoglobine in het serum. In deze studie tonen we aan dat locale haptoglobine expressie verhoogd is in artritis and kankerweefsel.

Er wordt algemeen aangenomen dat haptoglobine vooral in de lever wordt aangemaakt. Het is echter aangetoond dat ook andere weefsel dan de lever haptoglobin kunnen synthetiseren. Aangezien haptoglobine in hoge concentraties in het serum aanwezig is, is de functie van locale haptoglobine expressie nog niet bekend. In **hoofdstuk 5** onderzoeken we of veranderingen in glycosylering van haptoglobine de noodzaak van locale haptoglobine aanmaak in de vaatwand kan verklaren. Haptoglobine expressie en glycosylering werd bestudeerd in gedotterde konijnen. Dotteren leidde tot een verhoging van haptoglobine productie in de vaatwand in de eerste twee weken na beschadiging terwijl de expressie in de lever gelijk bleef. Er waren geen verschillen in glycosylering van haptoglobine in de vaatwand voor en na dotteren. Haptoglobine uit de vaatwand werd echter wel anders geglycosyleerd dan haptoglobine uit de lever en kon worden aangetoond in het serum.

Het is al eerder beschreven dat glycosylering van haptoglobine in het serum geassocieerd is met

het ontstaan en de prognose van bepaalde ziekten. In **hoofdstuk 6** wordt de potentiële rol onderzocht van haptoglobine glycosylering patronen in het serum als serum marker voor atherosclerose. Er blijken geen verschillen te zijn tussen totale serum haptoglobine concentraties en serum glycosylering van haptoglobine in patiënten met en zonder atherosclerose. Daarnaast is geen relatie aangetoond van totale serum haptoglobine concentraties en glycosyle-ring van haptoglobin met de intima-media dikte van de carotis, dat een maat is voor de mate van atherosclerose. Hieruit kunnen we concluderen dat de haptoglobine glycosyleringspatronen in het serum niet gebruikt kunnen worden als marker voor atherosclerose.
Hoofdstuk 7 is een algemene discussie over de resultaten van de voorafgaande studies.





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Dankwoord

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MIRJAM





Curriculum Vitae

Mirjam Smeets werd geboren op 3 juni 1974 te Geldrop. Het VWO diploma werd in 1993 behaald aan de Minkema scholengemeenschap te Woerden. Daaropvolgend begon ze in september 1993 met de studie Medische Biologie aan de Universiteit Utrecht waarvan het propedeuse werd behaald in 1995. Tijdens het doctoraal werden twee stages gelopen. De bijvakstage werd verricht bij de vakgroep Humane Genetica van het Universitair Medisch Centrum Utrecht. De hoofdvakstage werd verricht bij de vakgroep Virologie van de faculteit Diergeneeskunde van de Universiteit Utrecht. Het doctoraalexamen Medische Biologie werd behaald in 1998. Daarna begon zij in november 1998 in het Laboratorium voor Experimentele Cardiologie van het Universitair Medisch Centrum Utrecht met het onderzoek dat uiteindelijk heeft geresulteerd tot dit proefschrift. Vanaf januari 2003 is zij werkzaam als postdoc bij de vakgroep Parasitologie van het LUMC.





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