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 its 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. Tissue remodeling in arteries may induce a structural change in total arterial circumference and is a major determinant of luminal narrowing after balloon angioplasty, in atherosclerosis, and during sustained changes in blood flow. 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. 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 as well as arterial shrinkage after balloon angioplasty.

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 and restenosis after injury. Locomotion of SMCs requires newly synthesized collagen, implicating a mechanism whereby integrin clustering and linkage to the cytoskeleton are required for effective cell movement. 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 (Hoechst-Roussel Charles River, 3.5 kg). The untreated left femoral arteries served as control. Rabbits were anesthetized by intramuscular injection of methohexitol (0.15 ml) and Vetbasin (0.15 ml) followed by intravenous injection of epidural saline (1 mg/kg) and ventilation with N₂O/CO₂ (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 vein resulting in arterial enlargement. 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.

Rabbits were killed 1, 2 and 7 days after the operation. After 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 arteries, and serial sections (6 µm), spanning most of the vessel segment, were cut for several days before being embedded in paraffin. Eight or more parts distilled water). The right common carotid artery was ligated as described by Kumar and Lindner. 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 NIH Image software package.

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 (16 h) incubation at 4 °C in 15% sucrose in PBS. Samples were embedded in Tissue Tek (Menzel Glazer) 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 at a gelatinase concentration of 2 µg/ml. Each reaction contained 14 µl of cDNA, 200µM dNTP, 1 µM reaction buffer (Invitrogen) containing 1:80,000 CuSO₄/erythromycin (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 mRNA. 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'-AAAACACCTCTCTTGAAAC-3', reverse 5'-AACGACCTTCAAAACCTC-3'), mouse 18s (forward 5'-TCAAACACGCGAAAACCTAC-3', reverse 5'-ACCAGAACAAATCGCAATCC-3'), rabbit haptoglobin (forward 5'-GAACGACCTGGGAAAAGG-3', reverse 5'-TGAAAGATGCTGCGAGG-3'), rabbit 18s (forward primer 5'-TCGACACCGGAGAACTTCAC-3', reverse primer 5'-ACAAATGCTCAGCAAC-3').

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 with 0.5% acetic anhydride in 0.1 M TEA. Sections were subsequently washed twice in 2 x SSC for 5 min at RT, followed by 3 min in 2 x SSC/0.5% formamide at 37 °C.

For prehybridization, 100 µl of hybridization mix (50 % formamide, 1mg/ml RNA, 1 x Denhardt's, 10% destrane sulfate, 4 x SSC) was added to the slide and the slide was incubated 1 h at 60 °C. A Dig (1 µl)-labeled RNA probe was added, and hybridization continued at 60 °C. After hybridization, sections were washed in 0.1 x SSC at 45 °C for 15 min, followed by RNAase treatment (40 µg/ml RNAase 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 by transferring to 0.1 x SSC/0.1% Tween 20 at RT.
Genotyping was done as described earlier. MMP-2 or MMP-9 (Biogenesis) were spotted on the gel in different concentrations using the MMP-2 or MMP-9 activity assay (Amersham). The assay was performed according to manufacturer’s protocol.

**Immunohistochemistry**

Immunohistochemistry was performed with sections treated according to the aforementioned method and used after in situ hybridization. Sections were fixed for 10 minutes in acetone containing 0.03 % H₂O₂ 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-ri-ventien monoclonal antibody (Sigma) 1B4 at 4°C in PBS/0.1% bovine serum albumin (BSA). After 1B4, incubation, sections were rinsed in PBS (three times for 5 min) and were incubated with 1 µg/ml goat-anti-mouse peroxidase polyclonal antibody (DakoCytomation) 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 µg/ml 3-amino-9-ethylcarbazole substrate.

**Mouse primary fibroblasts**

Mouse primary fibroblasts were prepared as previously described. Mouse primary fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (InVitrogen) with 1% MEM nonessential amino acids (InVitrogen), 3.7 µg/ml β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS) (InVitrogen). Genotyping was done as described earlier.

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 lipoxydextran (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 chemotactrant according to manufacturer’s protocol. The cells were counted as migrated cells when they migrated and attached to the bottom well culture dish.

**Human primary 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 µg/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 CaCl₂, 0.05% Brij 35 (Sigma)) were used, in which F12 medium or 10⁻⁶ M haptoglobin (Sigma) or dextran (100 g/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. 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-2 activity was quantified by the amount of gelatin degradation (optical density/mm) using the Gel Doc 1000 system (Bio-rad). Zymography was performed as described earlier with 5 µl of medium from heterozygous or knockout murine embryonic fibroblasts.

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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 tissueblot (figure 2A) was used to determine relative amounts of haptoglobin mRNA in arteries and liver. This assay showed that 200 ng of total haptoglobin 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. When studied by using uncoated transwell culture chambers (Costar 3422) with approximately 20,000 cells per chamber, incubation of cells with 10 ng/ml lipoxydextran (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 chemotactrant according to manufacturer’s protocol. The cells were counted as migrated cells when they migrated and attached to the bottom well culture dish.

**Results**

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.

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Statistics
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\textsuperscript{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 than 100 times (p=0.0001) compared with its contralateral control artery (figure 2E, G). The haptoglobin mRNA levels in the right and left carotid artery were also increased more than 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-
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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 expression 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 (arbitrary 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.

Unilateral common carotid ligation in wild type and haptoglobin knockout mice

A murine carotid ligation model 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. 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. 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. Arteries from the 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

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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 2x10⁵ 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.
Figure 4. Arterial restructuring in a murine blood flow cessation model using wild type and haptoglobin knockout mice

A) Cross-sectional wall layer areas (mm²) 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). Intima + media (IMa), medial area (Media) and adventitial area (Adventitia) were measured and average values during the 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.
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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} M) or 1% FCS plus 10^{-8} M haptoglobin (10^{-8} M) haptoglobin (10^{-8} M) or 10^{-8} M haptoglobin (10^{-7} M) and 10^{-8} M haptoglobin (10^{-6} M) or 10^{-5} M haptoglobin (10^{-5} M). 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).

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^{-4} or 10^{-5} 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). 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^{-4} 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...
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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, 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. During hemolysis, however, haptoglobin knockout mice suffer greater renal tissue damage and fail to repair damaged renal tissue. 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 and abdominal aortic aneurysms are associated with elevated haptoglobin plasma concentrations. In vitro studies indicate a role for haptoglobin in bone resorption and inflammation. Moreover, haptoglobin stimulates angiogenesis both in vitro and in vivo and a haptoglobin-like mRNA may be associated with localized angiogenesis in human endometrium.

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\textsuperscript{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\textsuperscript{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\textsuperscript{32}. 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\textsuperscript{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\textsuperscript{35-37}, which we attribute to the prevention of gelatin formation by collagenases. Haptoglobin, in contrast, stimulates angiogenesis\textsuperscript{38} 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\textsuperscript{39} 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\textsuperscript{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
Haptoglobin is involved in arterial restructuring

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

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Haptoglobin is involved in arterial restructuring.