Blood flow-dependent arterial remodelling is facilitated by inflammation but directed by vascular tone

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Aims Altered blood flow affects vascular tone, attracts inflammatory cells, and leads to microvascular remodelling. We tested the hypothesis that inflammation facilitates the remodelling response, but that vascular tone determines its direction (inward or outward).

Methods and results Mouse mesenteric resistance arteries were ligated to create either increased blood flow or low blood flow in vivo. In vivo microscopy was used to determine changes in vascular tone. Structural remodelling was studied after 2 days, with or without macrophage depletion. In order to characterize the inflammatory response, immunostaining, confocal microscopy, and real-time PCR were used. To address the role of vascular tone in remodelling, arteries were treated with the vasodilator amiodpine during organ culture. Vessels exposed to high blood flow dilated, whereas low flow induced constriction. After 1 day, inflammatory markers showed a complex but remarkably similar increase in expression during high flow and low flow. Both high-flow and low-flow vessels showed an increase in the number of adventitial macrophages. Depletion of macrophages eliminated flow-induced remodelling. Manipulation of vascular tone reversed inward remodelling in response to low blood flow.

Conclusion Altered blood flow triggers an inflammatory response that facilitates remodelling. Vascular tone is a crucial determinant of the direction of the remodelling response.

KEYWORDS
Arteries; Vasoconstriction/dilation; Remodelling; Blood flow; Macrophages

1. Introduction
Vascular remodelling is associated with inflammation in a number of pathological conditions, including hypertension,1 aneurysms,2 and atherosclerosis.3 Also in the case of small artery remodelling in response to altered blood flow, inflammatory cells play a crucial role. More specifically, during arteriogenesis, increased shear stress attracts monocytes, which greatly enhance the formation of collateral arteries from small pre-existing vessels.4 These inflammatory cells may directly contribute to the remodelling response, through the release of matrix degrading enzymes such as matrix-metalloproteinases. While leukocytes may facilitate outward remodelling, we previously found that macrophages can also contribute to inward remodelling.5 In mice deficient for tissue-type transglutaminase, we observed that inward remodelling in response to reduced blood flow depends on perivascular macrophages. In these experiments, the cross-linking action of tissue-type transglutaminase, necessary for inward remodelling, was shown to be compensated by factor XIII from macrophages. Also in hypertension, a relationship between small artery inward remodelling and inflammation is recognized. Various models of hypertension are associated with vascular inflammation, probably related to the action of angiotensin II, endothelin-1, and the participation of oxygen radicals.1 Again, macrophages appear to play a causal role in vascular remodelling, since the inward remodelling associated with hypertension is absent in mice lacking proper macrophage function.6,7

Since inflammation is associated with both inward and outward remodelling of small arteries under different conditions, we hypothesized that inflammation participates in small artery remodelling in response to altered blood flow. Possibly, the nature of the inflammatory response differs in inward- vs. outward remodelling, thereby favouring a particular direction of remodelling. As an alternative possibility, we hypothesized that inflammation facilitates remodelling in general, but altered vascular tone, i.e. vasoconstriction or vasodilation determines the direction of the remodelling response. To test these hypotheses, we used the mesenteric artery ligation model in mice, which shows

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outward remodelling in arteries exposed to elevated blood flow, and inward remodelling in arteries exposed to flow cessation. In this study, we determined the changes in vascular tone shortly after ligation and structural remodelling after 2 days. Using quantitative real-time PCR and confocal microscopy, we characterized the inflammatory response during remodelling. Macrophage depletion was used to study the contribution of these inflammatory cells in remodelling. To test the role of vascular tone, we interfered with vasodilator treatment during the remodelling process.

2. Methods

2.1 Mesenteric artery ligation

Male and female mice (mixed C57/B16 and Svj background) of 4–5 months old were used for experiments. Procedures were approved by the local ethical committee. 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). Mice were anaesthetized with a mixture of ketamine/medetomidine/atropine (i.p.). After an incision through the abdominal skin and muscles, a loop of the intestine was exposed. From three adjacent second-order mesenteric arteries, the first and third arteries were ligated with 7-0 suture at the distal end of the vessels. This creates high flow in the middle vessel in addition to the two low-flow vessels. Control vessels were second-order mesenteric arteries obtained along the intestine, remote from the ligated vessels. These control arteries were isolated from the same mice at the same time-points. There was no loose ligation or other sham procedure for control or high-flow arteries. In a first set of experiments, images were obtained from arteries before and after 2 hours of altered blood flow. For this purpose, mice were placed on a heating pad while a loop of the intestine was placed on a sterile cloth. During the procedure, the intestine was kept moist with warm phosphate-buffered saline solution and heated with an infra-red lamp. Body temperature of the animals was monitored with a rectal probe. The exteriorized arteries were observed with a microscope and digitized images were obtained. The diameter of arteries was determined off-line with image J software. During the 2-hour interval between measurements, the intestine was placed back in the animal and the incision was provisionally closed. After the second measurement, animals were sacrificed and arteries were isolated. The maximal diameter of the observed arteries was then determined using a normalization procedure on a wire myograph. Based on the Laplace relationship (pressure=2 * tension/radius), this gives an estimated diameter at 100 mmHg. In all other experiments, the abdomen was closed directly after ligation with 7-0 sutures and the animals were allowed to recover. A subcutaneous injection with Temgesic (2 mg/kg) was given to reduce pain. After 1 day and 4 days, vessels were isolated and placed in Tri-buffer (4°C) for mRNA isolation; subjected to macrophage quantification. In a third set of experiments, the structural remodelling was determined after 2 days of altered blood flow. Arteries were isolated and cannulated in calcium-free MOPS buffer as described previously. Passive pressure–diameter relationships were determined. Remodelling is defined as a difference in the passive diameter at a given pressure between arteries from control and intervention groups. To study the role of macrophages, some animals were treated with liposome-encapsulated clodronate to suppress the peritoneal monocyte/macrophage population. Thus, clodronate containing liposomes were injected i.p. in two doses of 0.2 mL (1 day before surgery and during surgery). Clodronate containing liposomes were prepared as previously described. Clodronate was a gift of Roche Diagnostics GmbH, Mannheim, Germany.

2.2 Macrophage counts

Isolated mesenteric arteries were placed in formaldehyde. After washing in PBS, whole arteries were permeabilized in cold acetone and exposed to trypsin for antigen retrieval. Macrophages were stained with anti-CD68 antibody coupled to FITC (Serotec). Nuclei were stained with ethidium bromide. Vessels were viewed with a confocal microscope (Leica). Macrophages were counted and expressed as number/mm vessel length.

2.3 Real-time PCR

Mesenteric small arteries were dissected in MOPS buffer at 4°C and kept in 1 mL Tri-reagent (Sigma) for mRNA extraction. cDNAs were subsequently synthesized using an Omniscript reverse transcriptase kit (Qiagen). Quantitative real-time PCR was performed in IQ SYBR® Green Supermix buffer (Invitrogen) using a MyiQ (Biorad) thermal cycler. Primer sequences were designed using Beacon software. Primer sequences: CD68, FW: 5'-GGACTACATGCGGTTGAAATAC-3' and RV: 5'-GAGGACGGTCGAACTGCAAG-3'; CD11b, FW: 5'-CATCAATAGCGCCTCCTACG-3' and RV: 5'-GGTTGCCCTCCAGTCTCAG-3'; TGFB1, FW: 5'-TGAACGAGGAGACGGAATACAG-3' and RV: 5'-GCCATGAGGACGAGGAAGG-3'; IL-10, FW: 5'-TGCCTGCTGCTCTTACCG-3' and RV: 5'-GCAATTAGGAGCTGGTAGC-3'; IL-4, FW: 5'-TACGGTGTGCTCGTCTCCTC-3' and RV: 5'-GGGTGTGCTTGTTGCGGTG-3'; TNFa, FW: 5'-GGCAGCGACCGAACCAG-3' and RV: 5'-ACAGCCAAGTGAAGGAGG-3'; and ribosomal phosphoprotein P0 as house-keeping gene, FW: 5'-GGACCGGAGAGACCTCCTT-3' and Rev: 5'-CACATACTCGAGAATTTCAATGG-3'.

2.4 Organ culture

Organ culture of isolated mesenteric arteries was performed as described previously. Briefly, vessels exposed to low blood flow were isolated 24 h after surgical intervention. Perfusion flow was set at zero during organ culture, to maintain the low-flow condition. During the following 24 h culture period, vessels were kept in Leibovitz medium (Gibco) supplemented with amloidipine (10^{-7} M) to maintain vasoaddition as described previously. Medium with amloidipine was pumped through the vessel chamber continuously. Pressure was set to 80 mmHg, and temperature was kept at 37°C. At the end of the culture period, the passive pressure–diameter relationship determined after full dilation with amloidipine (10^{-7} M).

2.5 Statistics

Results are presented as average ± standard error of the mean. Averages were compared using Students’ t-test or ANOVA followed by Dunnett’s t-test with equal or unequal variances as appropriate. Differences were considered statistically significant at P < 0.05.

3. Results

3.1 Effect of altered blood flow on vascular tone

The effect of surgically modified blood flow on mesenteric arterial diameter was determined using in situ observation. Measurements of arterial diameter were made before and 2 hours after the flow intervention. The average in vivo diameter of the observed arteries before flow intervention was 193 ± 9 μm (n = 12). Low flow induced a significant decrease in arterial diameter of 17 ± 5% (n = 6). In contrast, the high-flow artery located between the two ligated arteries showed a significant increase in diameter of 17 ± 7% (n = 6). Figure 1 shows the average data. After the measurements, the vessels were isolated from the tissue and mounted in a wire myograph to determine the
maximal diameter. This yielded a diameter of 244 ± 9 μm for low flow and 235 ± 23 μm for high flow (NS). Although not the focus of this study, with ligation also a marked decrease in diameter of the paired vein was observed. Thus, the average venous diameter decreased with 59 ± 3% (n = 4; P = 0.002). The diameter of the paired vein of the high-flow artery did not significantly change.

3.2 Macrophage accumulation

In the next series of experiments, the recruitment of macrophages to the arteries was studied. Macrophages were identified by immunostaining and confocal microscopy. Vessels were isolated 24 h after modified blood flow and compared to control vessels from the same animals (Figure 2A). The macrophages were all located on the adventitial side, distributed along the length of the artery (Figure 2B). Occasionally, small groups of macrophages were observed. On average, both arteries exposed to low blood flow and high blood flow showed a 5-fold increase in the number of macrophages (Figure 2C).
3.3 Remodelling and macrophage depletion

To study structural remodelling, i.e. a change in passive diameter of the arteries, vessels were isolated after 2 days of altered blood flow. A pressure–diameter relationship of control, low-flow, and high-flow arteries was determined (Figure 3A). A significant difference in passive diameter between low-flow and high-flow arteries was observed at higher pressure levels, indicating flow-induced remodelling. Animals that were treated with liposome-encapsulated clostronate to eliminate macrophages showed no difference in passive pressure–diameter relationships among arteries (Figure 3B), demonstrating that macrophages play a causal role in flow-induced outward and inward remodelling.

3.4 Inflammatory response to altered blood flow

To characterize the phenotype of the inflammatory response during flow-induced remodelling, the expression of a number of inflammatory markers was determined by qRT-PCR. Arteries were harvested 1 day and 4 days after modified blood flow. Overall, a profound increase in the expression of genes related to inflammation was found after 1 day. After 4 days, however, most of the inflammatory cytokines were barely detectable and expression was not significantly different from control vessels (Figure 4). After 1 day, the expression of CD68, a macrophage marker, was similarly increased in low-flow and high-flow vessels. Also CD11b, a marker of activated leukocytes, was up-regulated in both low-flow and high-flow vessels. The expression of tumour necrosis factor α (TNFα) and interleukin-1β, both cytokines associated with a T-helper-1 (Th1) inflammatory response, showed a similar increase in expression with low flow and high flow. The expression of interleukin-4, a cytokine associated with T-helper-2 lymphocytes (Th2), was significantly up-regulated in low-flow vessels, but did not reach statistical significance with high flow (P = 0.07). A marker of Th2-activated macrophages, the plasma transglutaminase, factor XIII, was unchanged. The inflammatory response was not only characterized by an up-regulation of pro-inflammatory cytokines, but also accompanied by increased expression of anti-inflammatory cytokines TGFβ and interleukin-10.

In both low flow and high flow, there was a more than 10-fold increase in the expression of matrix-metalloproteinase 1 (MMP-1). However, due to a large variation among experiments, these data did not reach statistical significance. Matrix-metalloproteinase 9 (MMP-9) was significantly up-regulated with low flow (Figure 4). With high flow, the MMP-9 expression increased ~100-fold, but did not reach statistical significance due to a large variation among individual experiments.

3.5 Manipulation of remodelling during organ culture

The sequence of events from vascular tone, inflammation, to remodelling prompted us to further study the relationship between vascular tone and remodelling. Vessels exposed to low blood flow in vivo (Figure 3A) were compared to vessels that were isolated after 1 day low flow, and subsequent in vitro exposure to the vasodilator amlodipine for another 24 h. During this in vitro period, the flow was kept zero. Maximal diameters were determined to reveal structural remodelling (Figure 5). Control vessels from both groups were similar: 223 ± 10 vs. 233 ± 15 μm (at 80 mmHg) and therefore pooled. Treatment with amlodipine reversed the direction of remodelling in response to low blood flow from inward to outward remodelling.

4. Discussion

The architecture of the mesenteric circulation consists of an arcading network, which allows the surgical modification of blood flow without concurrent tissue ischaemia. Redistribution of blood flow is accompanied by relatively fast arterial remodelling in rats and mice, and occurs within 2–7 days. The current study shows that remodelling (day 2) is preceded by fast changes in vascular tone (within 2 h), followed by an inflammatory response (day 1). The inflammatory response is not a general effect of the surgery itself, as control vessels were obtained from the same animal at the same time-point. Rather, these data suggest that changes in blood flow, both up- and downward, trigger an inflammatory response. This inflammatory response is
characterized by recruitment of macrophages, and increased expression of both pro- and anti-inflammatory cytokines. The timing of the inflammatory response suggests that it coincides with the initiation of remodelling (Figure 6A). Thus, all inflammatory markers had returned to baseline values after 4 days. These data are in agreement with a study in rats, where micro-array analysis was performed at various time-points during the remodelling response. This revealed that among the early responsive genes, many were related to the immune system.18

4.1 Phenotype of inflammation

Immunostaining and mRNA expression of CD68 showed that macrophages were attracted to the remodelling arteries. The integrin CD11b is expressed by monocytes/macrophages and other types of leukocytes, and is necessary for cell adhesion and migration. Both markers were similarly up-regulated in high flow and low flow. As indicated earlier, macrophages play an important role in the inward remodelling of small arteries in hypertension, and outward remodelling of small collateral arteries in response to increased shear stress. In addition, our previous work indicated that macrophages can participate in the inward remodelling induced by reduced blood flow in mice deficient for tissue-type transglutaminase.5 Albeit relatively low numbers of macrophages were observed, in the present study we found that treatment with liposome-encapsulated clodronate prevents flow-dependent remodelling. These results, therefore, show that macrophages play a causal role in the remodelling. We did not determine the expression of inflammatory markers after treatment with clodronate; further studies would be needed to elucidate this point.

One of our initial thoughts was that the phenotype of the macrophage19 may differ in outward vs. inward remodelling vessels. Thus, a lytic phenotype stimulated by Th1 cytokines (IL-1β, TNFα, IFNγ) could facilitate outward remodelling, whereas a fibrotic phenotype induced by Th2 cytokines20 (IL-4, IL-13) could participate in inward remodelling. For instance, l-arginine can be used by macrophages to form nitric oxide and citrulline when stimulated by Th1 cytokines, but can also be converted to ornithine and used for collagen synthesis when stimulated by Th2 cytokines.20 One of the markers of such Th2 stimulated macrophages is the plasma transglutaminase, Factor XIII.21 In our previous work, we found that Factor XIII, a cross-linking enzyme, is indeed involved in inward remodelling after reduced blood flow in type 2 transglutaminase knock-out mice.5 The results obtained in the present study, however, do not show a clear difference in the phenotype of the inflammatory response in high-flow vs. low-flow vessels. Instead, a complex profile of both pro- and anti-inflammatory cytokines was observed after 1 day, which was similar in high flow and low flow. After 4 days, most of the cytokines had returned to baseline expression levels. Although it cannot

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Figure 4 RT-PCR analysis of inflammatory markers and matrix metalloproteinases. Expression of CD68, CD11b, IL 4, Factor XIII, TNFα, IL 1β, TGFβ, and IL 10 was determined in whole vessels at day 1 (n = 6 or 7) and day 4 (n = 3) after altered blood flow. Expression of MMP-1 and MMP-9 was determined at day 1 only. Mesenteric arteries were exposed to control flow (control), low blood flow (LF), or high blood flow (HF). Expression is normalized to the housekeeping gene. The level of statistical significance is indicated by *P < 0.05, **P < 0.01, or ***P < 0.001.
be excluded that other cytokines show a difference in expression between low flow and high flow, these data therefore support the hypothesis that inflammation plays a role in the initiation of remodelling rather than determine the direction of the remodelling.

A key step in vascular remodelling is the activation of metalloproteinases. Inflammatory cytokines could up-regulate the expression of metalloproteinases, and thereby contribute to a state of plasticity through partial degradation of matrix proteins, cell–cell and cell–matrix connections. Here we studied the expression of MMP-1 and MMP-9. The latter has previously been identified as a crucial enzyme in remodelling of mouse carotid arteries and small rat mesenteric arteries. Our results suggest that MMP expression is not exclusively expressed during outward remodelling, but also up-regulated during inward remodelling. Intervention studies are needed to pinpoint the importance of these and other matrix metalloproteases. In addition, the source of MMP expression in the vessel wall remains to be established. Although zymography suggests that smooth muscle cells express MMP-9 in the intact vessel wall, it is also possible that leukocytes are an important source themselves. Albeit in human cultured cells, MMP-9 expression was found to be 105-fold higher in macrophages when compared with vascular smooth muscle cells.

4.2 Vascular tone and remodelling

In situ observation of the arteries before and after ligation showed that arteries adapt their level of tone in response to modified blood flow. The phenomenon of flow-dependent
The regulation of vascular tone is well established, and depends on the release of dilatory factors from the endothelium. \(^{27}\) In the current experiments, the 17% increase in vessel diameter of high-flow arteries would accommodate an 87% increase in flow according to the Poiseuille equation. In previous work, we showed that chronic changes in vascular tone trigger vascular remodelling in the direction set by the level of vascular tone. \(^{11,28,29}\) Thus, chronic vasoconstriction induced inward remodelling in several types of arteries, whereas chronic vasodilation caused outward remodelling in porcine coronary arteries. These findings are further supported by in vivo data obtained by Eftekhari et al. \(^{30}\) These authors showed that infusion of vaso-constrictor and -dilator substances in rats induce inward and outward remodelling, respectively. The current study shows that initial changes in tone are followed by structural remodelling after 2 days in mouse mesenteric arteries. We therefore hypothesized that the changes in vascular tone observed in the current experiments are a critical determinant in the remodelling response. To further substantiate this, we isolated low-flow arteries 1 day after ligation and placed these in organ culture for another day. While flow was kept zero in vitro, vasodilator treatment completely reversed the direction of remodelling. Thus, vessels exposed to low blood flow in vivo showed a decrease in diameter, while in vitro treatment of low-flow vessels results in an increase in diameter. These experiments, therefore, show that the direction of the remodelling can be reversed by manipulation of vascular tone.

Taken together, the results of the current study suggest that inflammation facilitates remodelling, possibly related to the expression of matrix metalloproteinases. However, vascular tone determines the direction of the remodelling response. A schematic representation of this view is shown in Figure 6B. Further work, however, is needed to establish whether this concept is relevant for pathological conditions associated with inflammation and remodelling, such as hypertension, atherosclerosis, and aneurysm formation.

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