Smooth Muscle Cell Changes During Flow-Related Remodeling of Rat Mesenteric Resistance Arteries


Abstract—To obtain information on the molecular and cellular mechanisms of flow-induced arterial remodeling, we analyzed the morphology and smooth muscle cell (SMC) characteristics in rat mesenteric resistance arteries after interventions that decreased and increased flow. Juvenile male Wistar Kyoto rats were subjected to surgery that, compared with control arteries, provided arteries with chronic low flow and chronic high flow. Low flow resulted in a decreased passive lumen diameter, hypotrophy of the artery wall, and both loss and decreased size of SMCs. Time course studies, with intervention length ranging from 2 to 32 days of altered blood flow, showed that the narrowing of the lumen diameter in low-flow arteries appeared within 2 days and that an early dedifferentiation of SMC phenotype was indicated by markedly reduced levels of desmin mRNA. High flow resulted in an increased passive lumen diameter and in hypotrophy of the artery wall. The hypertrophy resulted from SMC proliferation because SMC number, measured by the 3D-dissector technique, was increased and immunohistochemical assessment of proliferating cell nuclear antigen also showed an increase. The widening of high-flow arteries required 16 days to become established, at which time desmin mRNA was reduced. This time was also required to establish changed wall mass in both low-flow and high-flow arteries. Apoptotic cells detected by TdT-mediated dUTP-biotin nick end labeling staining were mainly located in the medial layer, and evaluation of DNA fragmentation indicated that increased apoptosis occurred in both low flow and high flow. This study shows for the first time direct evidence that reduced and elevated blood flow in resistance arteries produce, respectively, decrease and increase in SMC number, with dedifferentiation of the SMCs in both cases. (Circ Res. 2001;89:180-186.)

Key Words: rat ■ vascular remodeling ■ blood flow ■ small arteries

Mechanical forces related to the velocity of the arterial blood flow are important factors determining the caliber of arteries.1,2 Acutely, the artery caliber responds to altered blood flow through the change in shear stress detected by the endothelial cells, which modulates the release of vasoactive factors.1 These factors may also have a role in long-term structural changes of arteries exposed to chronically altered blood flow,3 as may the altered circumferential wall stress caused by the initial functional adaption to chronic changes in blood flow (eg, Ben Driss et al4).

Flow-related remodeling of resistance-sized arteries is important for maintaining an adequate tissue perfusion to allow the structural set point to be optimal for acute vasomotor responses. Remodeling of skeletal muscle arteries during endurance training and remodeling of uterine arteries during pregnancy are examples of arterial remodeling that are due to increased blood flow.5,6 Also, in diseased states, hemodynamic forces are implicated in the remodeling of, for instance, coronary collateral arteries after ischemic injury to the heart.7,8 Experimental analyses of flow-induced remodeling in large arteries have primarily involved unilateral carotid artery ligation in mice, rats, and rabbits, showing a flow-dependent decrease of lumen diameter in the ligated artery and an increase of diameter in the contralateral artery.9-11 Similarly, the small arteries of the mesenteric bed of the rat have been used for the in vivo study of flow-induced remodeling, where ligation of feed arteries in this vascular bed acutely and chronically results in increased blood flow through collateral pathways. Unthank et al12 showed lumen enlargement and medial hypertrophy in the collateral pathway, and Pourageaud et al13 established a model to study both inward hypotrophic and outward hypertrophic remodeling in response to chronically decreased and increased blood flow, respectively.

The cellular mechanisms producing the remodeling remain unclear. The altered morphology could be due to changes in size and arrangement of existing SMCs or to combinations of cell proliferation and cell death (apoptosis). Furthermore, it is
not clear if the remodeling is associated with SMC dedifferentiation. To address these questions, we have used the ligation model of rat mesenteric arteries during 2 to 32 days of blood flow manipulation. We found that the outward remodeling due to high flow was a slow process compared with the quick inward remodeling noted in chronically hyperperfused arteries. In both cases, the flow-related arterial remodeling involved changes in cell turnover and cell phenotype.

Materials and Methods

Animals and Surgery

Male Wistar Kyoto rats (animal facilities, Universiteit Maastricht) of 6 to 8 weeks of age were used for the experiments. Blood flow-modifying surgery was performed in the animals by distal ligation of alternate first-order mesenteric arteries to give low flow (LF); the patent arteries between these then had a compensatory high flow (HF).13,14 Arteries were taken from a point proximal to the ligation (LF artery) and from a corresponding position in the patent arteries (HF arteries). For the stereology experiments and assessment of phenotype markers by immunohistochemistry, control arteries were taken from animals where a sham surgical procedure was performed but without blood flow modification.13 In the other experiments, control arteries were taken from the same animal as the flow-modified arteries as described by Ceiler et al.13 These interventions give, respectively, ~80% decrease and an approximate doubling of flow in LF and HF arteries, but with similar intravascular pressures.13,14

Stereology

Arteries segments isolated at 4 weeks after surgical intervention were mounted in an isometric myograph.14 The artery was exposed to a resting wall tension corresponding to a transmural pressure of 100 mm Hg.15 Processing of the tissue and determination of the volume and density of the cells and nuclei in the medial layer were performed as described by Korsgaard and Mulvany.16

Pressure-Diameter Relation

Arteries were mounted in an organ chamber (Living Systems) filled with calcium-free Krebs-Ringer bicarbonate (KRB, 37°C, see below) aerated with 95% O2/5% CO2. The proximal end of the artery was mounted on a cannula and the other end was ligated as a blind sack14 after flushing the lumen with calcium-free Krebs-Ringer bicarbonate (KRB, 37°C, see below). The composition of the KRB buffer was (in mmol/L) NaCl 119, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, and glucose 5.5. In calcium-free KRB buffer, CaCl2 was omitted from the solution. The composition of the PBS solution was (in mmol/L) NaCl 150, Na2HPO4 11, and KH2PO4 1.6.

Polymerase Chain Reaction (PCR) Amplification of Desmin and GAPDH mRNA

Total RNA was extracted as described previously17 and 100 ng RNA was reverse-transcribed (RT). PCR was performed with primers for either desmin cDNA (5'-CGAGAGCTGGCGGCCAAAGT-3', forward; 5'-CATCGTTGTCTATTGGCTGCG-3', reverse) or GAPDH cDNA (5'-ACGGATTGGCGCCGAAGT-3', forward; 5'-CGTCAGATCCACGACGGA-3', reverse). The products were size-fractionated in a gel containing 1.5% agarose, and the intensities of the bands were quantified by densitometry.

Extraction of DNA

DNA extraction was based on the Wizard genomic DNA purification kit from Promega. DNA content was determined by the fluorometric method of Labarca and Paigen with calf thymus DNA (Sigma Chemical Co) as standard.

Ligation-Mediated PCR of Blunt-End DNA Fragments

Ligation-mediated PCR, performed to amplify DNA fragments characteristic for apoptotic cell death,9,20 was based on the ApoAlert LM-PCR ladder assay kit (Clontech). PCR amplification was performed with 75 ng of ligated DNA. For each ligation procedure, the number of cycles needed to detect laddering in the calibration sample, consisting of a batch of rat thymus DNA, was determined and related to the laddering of artery samples. A score was applied to the samples (laddering index): 2 points were given if laddering was detectable with the number of cycles giving visible laddering for the calibration rat thymus sample, 1 point was given if laddering was detectable with an additional 5 to 6 cycles, and 0 points were given if laddering was not detectable after these additional 5 to 6 cycles.

Morphometry and Immunohistochemistry

Cross sections (4 μm thick) of arteries fixed at a transmural pressure of 100 mm Hg were used for the determination of medial cross-sectional area (CSA) and for assessment of phenotypic markers by immunohistochemistry. CSA of the medial layer was defined as the area between the internal and external elastic laminae. Cross sections used for immunohistochemistry were incubated overnight with different primary antibodies/antisera as indicated. The chromogen for the color development was 3,3'-diaminobenzidine (DAB), and sections were lightly counterstained with hematoxylin. These stainings were evaluated blindly by two independent observers. The sections were scored according to 4 levels of intensities: from 0 if no stain was observed to 3 if the entire medial layer was stained.

Longitudinal sections were used for detection of proliferating cell nuclear antigen (PCNA, clone PC10, DAKO). Each nucleus was determined to be either positive (brown) or negative (blue), and at each time point the values were normalized to the mean of the control, which was given the value of 1. Apoptotic cells were detected by using the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method on longitudinal sections. The sections were treated with 3% citric acid and then with proteinase K (10 μg/mL in PBS) before incubation with TdT enzyme (300 U/mL, Promega) and biotin-16-dUTP (0.02 nmol/L, Boehringer Mannheim). Development of color was as described for the above procedures. Apoptotic cells were identified when both TUNEL staining and karyorrhexis were observed.

Solutions

The composition of the KRB buffer was (in mmol/L) NaCl 119, KCl 4.7, CaCl2 2.5, MgSO4 1.2, NaHCO3 25, KH2PO4 1.2, and glucose 5.5. In calcium-free KRB buffer, CaCl2 was omitted from the solution. The composition of the PBS solution was (in mmol/L) NaCl 150, Na2HPO4 11, and KH2PO4 1.6.

Statistical Analysis

To test differences of mean values between control arteries and arteries exposed to altered blood flow (LF and HF) we used unpaired Student’s t test, paired Student’s t test, or the nonparametric tests Mann-Whitney U test and Wilcoxon signed-rank test, as indicated. Groups were considered significantly different when P<0.05.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Flow-Induced Structural Changes

Rats (n=30) at 8 weeks of age were exposed to flow-modifying surgery, and arteries were isolated for analysis at days 2, 4, 8, 16, and 32 after intervention. The morphological changes are shown in Figure 1A. After as little as 2 days, a significantly decreased lumen diameter was observed for the arteries exposed to LF. After this initial narrowing, further narrowing was especially seen after day 16. Arteries exposed...
Table 1, stereological analysis of the SMC number and size was performed to determine the number and size of the SMCs in the medial layer. Values represent mean±SEM.

*P<0.001, †P<0.05 vs control arteries, two-tailed Student’s t test.

Arteries kept for 4 weeks at altered blood flow conditions were isolated, mounted in an isometric myograph, and stretched to a level corresponding to a transmural pressure of 100 mm Hg. The arteries were then treated with fixative, sectioned, and stained, and the 3D-dissector counting technique was performed to determine the number and size of the SMCs in the medial layer.

The 3D-dissector results presented in Table 1 show an increased number of SMCs in the medial layer of chronically hyperperfused arteries. To investigate if this was associated with increased SMC proliferation, immunohistochemical detection of PCNA (a nuclear protein abundant in cells in the S-phase of the cell cycle) was determined. PCNA overall showed higher counts in hyperperfused arteries, consistent with medial hypertrophy being associated with increased SMC proliferation. The PCNA counts were elevated (∼100%) in two phases of the time course, first at day 8 and then later at day 32 (Figure 2). There were no statistically significant differences between LF arteries and control arteries, but the level of PCNA immunoreactivity fluctuated around the basal level with the same biphasic appearance as seen for the HF arteries.

**SMC Phenotype**

In control, LF, and HF arteries, markers of SMC phenotype were assessed by immunohistochemical stainings 4 weeks after flow manipulation (Table 2). Smooth muscle α-actin and smooth muscle myosin heavy chain showed no detectable changes in response to altered blood flow at this time point. The thin filament–associated proteins h-caldesmon and calponin, and the intermediate cytoskeletal protein desmin showed no change in arteries exposed to HF. However, the level of desmin and calponin protein was reduced in the LF arteries (Table 2), indicating a dedifferentiation of the SMCs. At the mRNA level, desmin also showed a marked decrease at both 4 and 16 days after surgery in LF arteries (Figure 3). At day 16, desmin mRNA expression was also moderately suppressed in HF arteries (Figure 3), which is in line with the nonsignificant tendency to a decrease of the protein levels after 4-week exposure to elevated blood flow (Table 2).

**Apoptosis**

Figure 4A shows the amplification of control tissue (rat thymus) DNA at 22 cycles and arterial samples (control, LF,
and HF, from days 4 and 16 after intervention) amplified for
an additional 6 cycles (28 cycles). DNA fragmentation is
present in both LF and HF arteries in these two animals,
which is indicative for the general finding that apoptosis
appeared higher in flow-manipulated arteries compared with
control arteries. Semiquantitative analysis of the material (see
Materials and Methods) confirmed that apoptosis was in-
creased in both LF and HF arteries compared with control
(araries (Figure 4B). Longitudinal sections from four day 16 animals
were subjected to TUNEL analysis combined with observa-
tion of karyorrhexis, providing evaluation for apoptosis for a
total of 12 110 nuclei. We detected 11 positive nuclei, of
which 9 were located in the medial layer (Figure 5) and 2 in
the adventitial layer of the arterial wall. Of these, 5 of the 11
apoptotic nuclei were found in the wall of LF arteries (total of
2740 nuclei), 2 in HF arteries (total of 3940 nuclei), and 4 in
control arteries (total of 5430 nuclei). Clustering of apoptotic
cells was not observed.

**Discussion**

The results of the present study provide direct evidence that proliferatio
}\cell loss, and phenotypic changes of the SMCs are involved in the remodeling response to altered blood flow
in rat mesenteric small arteries.
In line with earlier observations, chronic reduction of flow ultimately resulted in a structural narrowing of mesenteric arteries of the rat, whereas increased flow resulted in a widening. These alterations represent remodeling rather than a reduced or accelerated growth of the arterial wall, because control arteries did not change their structure (Figure 1), as also found previously. The changes seen in LF and HF arteries have previously been demonstrated to normalize wall shear rate and circumferential wall stress after 4 weeks of hypoperfusion or hyperperfusion. At least two main mechanisms for these normalizations can be hypothesized: (1) Flow-induced changes of vasomotor tone or structural lumen diameter cause changes of the tensile forces in the artery wall, which is then normalized by either wall hypertrophy or hypotrophy. (2) Signaling substances liberated or suppressed due to altered flow affect both lumen caliber and artery wall mass, resulting in a tight connection between these parameters. In the present study, both mechanisms may be represented. Evidence for tensile force being a signal comes from our finding in LF arteries that the structural narrowing preceded the hypotrophy of the artery wall by several days (Figure 1). On the other hand, the finding in HF arteries that final stages of widening and hypertrophy of the arterial wall were established simultaneously by day 16 after the intervention (Figure 1), as also found by Tulis et al in a similar system, is more consistent with the presence of a signaling substance. Whether this difference in inward and outward remodeling indeed results from totally different mechanisms or is because of the ~80% reduction in flow is a stronger signal to the arteries than a doubling in flow needs to be investigated further.

Although the model used in the present study is designed to investigate the effects of changes in flow, the vessels examined may also have been exposed to slight changes in pressure. Thus, under control conditions, the pressure in the vessels will be ~10 mm Hg below the pressure in the superior mesenteric artery. The ligation process would be expected to cause the pressure to rise slightly in the LF arteries as a result of the 80% decrease in flow, the rise, however, being substantially offset by the increase in the hydrodynamic resistance of the arteries because of the decrease in lumen diameter of the vessels. Conversely, in the HF arteries, the ligation procedure could cause a slight decrease in pressure, the rise being offset by the decreased hydrodynamic resistance due to the increase in lumen diameter. Thus, any changes in pressure in LF and HF arteries were probably small.

The rapidity of the narrowing of the LF arteries, which occurred within 2 days, was surprising, particularly because this narrowing did not progress much further when the intervention period was prolonged to up to 32 days. The lumen diameter of the arteries was measured in vitro at 100 mm Hg in a calcium-free solution, which suggests that the narrowing results were not due to contraction. It cannot, however, be excluded that the narrowing is due to a prolonged calcium-independent rigor, as seen when certain smooth muscle preparations are exposed to protein kinase C activators (eg, see Walsh et al). Furthermore, it has been shown that lowering shear stress causes higher expression of endothelin 1 in endothelial cells (eg, Malek et al) and that endothelin 1 is able to induce a stable protein kinase C–dependent contraction of rat aorta strips in a calcium-free Krebs buffer. Recently, inward remodeling was shown for resistance arteries from rat cremaster muscle kept in organ culture for 4 days at 75 mm Hg in the absence of flow. These authors suggested fibroblast growth factor-2 (FGF-2) as a candidate mediator of the inward remodeling, based on observations by Bryant et al that anti–FGF-2 antibodies are able to inhibit the inward remodeling in the unilateral carotid artery ligation model in mice. Persistent contraction induced by FGF-2 may be converted into a reorganization of the cytoskeleton of arterial SMCs and endothelin 1 (see above), or a combined activity of these components could thus be the signals for the rapid inward remodeling.

In the LF arteries, wall hypotrophy was established after 16 days, and after 4 weeks of hypoperfusion, a marked loss of SMCs was observed by using the 3D-dissector technique (Table 1). Our biochemical and histological evidence indicates that apoptosis is involved. This is consistent with the findings of Cho et al showing increased levels of nucleosomal ladders after reduction of the blood flow in the common carotid artery of the rabbit. Furthermore, our data showed that arteries hypoperfused for 4 weeks not only had fewer cells but the remaining cells were considerably smaller than those in control arteries. Cell volume regulation then has to be included as a significant factor for the remodeling, and it would have been interesting to see to what extent volume regulation and cell loss contribute to the decrease of CSA at the earlier time points, especially because cell shrinkage is one the characteristics of a cell in apoptosis. Furthermore, with a 67% decrease of cell number and a 40% decrease of the mean cell volume, hypotrophy of the hypoperfused arteries is primarily a cellular phenomenon rather than as a result of extracellular matrix degradation. A reduced proportion of the cellular compartment of the arterial wall in LF arteries might be responsible for this marked stiffening as indicated by steeper stress-strain relationships.
The widening of the HF arteries, which reached a plateau at day 16 after introduction of the flow modification (Figure 1A), is in line with earlier results with the model showing significant widening after 4 weeks\textsuperscript{13} and 2 weeks\textsuperscript{14} of intervention. It is, however, delayed compared with the 7 days required for reaching a significant widening in a similar ligation system of rat mesenteric arteries analyzed by Tulis et al.\textsuperscript{22} With regard to the hyperperfusion-induced increase in CSA accompanying the widening of the arteries, two lines of evidence in the present study indicate that this is associated with proliferation. First, the 3D-dissector analysis of arteries hyperperfused for 4 weeks showed a 44% increase of the number of SMCs per artery length with no change in cell volume (Table 1). Second, the marker for SMCs in S-phase was elevated in hyperperfused arteries, where the time course of the PCNA levels showed a biphasic pattern with peaks at days 8 and 32 (Figure 2). The peak at day 8 preceded the increase in CSA, consistent with the expression of PCNA preceding hypertrophy compared with CSA also observed by Tulis et al.\textsuperscript{22} although this appeared at an earlier time point (days 3 through 7). The late peak of PCNA seen at day 32 could be related to a later phase of remodeling appearing beyond 4 weeks, as observed by Fath et al.\textsuperscript{32}

A regulation of the balance between cell proliferation and cell death may be the most important mechanism for remodeling, as has been suggested for embryonic development and normal tissue homeostasis (for review, see Best et al.\textsuperscript{33} and Hamet et al.).\textsuperscript{34} This is supported by the present study, where apoptosis determined by DNA laddering was more frequent in both the LF and HF arteries compared with control (Figure 4B). The intensity of the laddering was rather variable, which was also observed by Cho et al.\textsuperscript{31} using a similarly sensitive laddering-detection technique in carotid arteries of rabbits. It was also of interest that TUNEL staining (Figure 5) did not detect clusters of cells, confirming apoptosis as a single cell event.

An increased level of apoptosis in hypertrophic arteries might seem inconsistent with a balance changed toward cell proliferation. However, in spontaneously hypertensive rats treated with N\textsuperscript{2}-nitro-L-arginine methyl ester, an increased TUNEL count was previously observed in hypertrophic coronary artery smooth muscle.\textsuperscript{35} Also, in growing coronary collateral arteries, an increased apoptosis was observed\textsuperscript{36} and in deoxycorticosterone acetate (DOCA)-salt hypertensive rats, an increased CSA was associated with an increased laddering intensity.\textsuperscript{35} As discussed by Hamet at al.\textsuperscript{34} “there is more than a conceptual link between apoptosis and its physiological counterpart, cell proliferation” (page 854). It is even suggested that apoptosis could act as a stimulus for wall hypertrophy. Indeed evidence for apoptosis as a normal physiological process comes from our findings of low but detectable levels of apoptosis in control arteries. This is consistent with previous findings, where a significant level of DNA synthesis was observed in 6-week-old rats, although there was no increase in DNA content by 20 weeks of age.\textsuperscript{36} Taken together, this suggests that there is a basal but slow rate of cellular turnover in intact, structurally stable, small muscular arteries, a turnover that is increased under conditions of altered flow.

SMCs have the ability to modulate their phenotype within a certain range from differentiated contractile type to the synthetic phenotype (for review, see Owens\textsuperscript{37}). In the present study, the level of the smooth muscle markers desmin and calponin investigated by immunohistochemistry showed a decrease in LF arteries, whereas the presence of smooth muscle α-actin and smooth muscle myosin heavy chain was hardly affected. Also, the levels of desmin mRNA were markedly decreased as early as day 4, which persisted at day 16. Thus, dedifferentiation of the contractile SMCs was pronounced and had an early onset. This dedifferentiation could be due to the early narrowing of the artery that at the unchanged transmural pressure results in decreased wall stress. Such a direct role for mechanical factors was seen in organ culture of rabbit aorta, where application of pressure (wall stress) prevented the decreased expression of smooth muscle differentiation markers seen in the isolated artery.\textsuperscript{38} In the HF arteries, when desmin mRNA is taken as a differentiation marker, a dedifferentiation was also seen with a later onset and also the immunohistochemical analyses showed the same tendency. The reason for this dedifferentiation could be completely different from the one seen in LF arteries, because proliferation of SMCs has been linked to a conversion of the cells to more synthetic types (see Owens\textsuperscript{37}).

In conclusion, this work provides the first direct evidence concerning the time course of and cellular basis for the changes in resistance artery morphology in response to decreases and increases in flow. The structural response to reduced blood flow consists of a rapid reduction of lumen diameter that is followed by medial hypotrophy and cell loss. The response to increased blood flow is a slow and parallel increase in lumen diameter and medial mass, where at least the increased medial mass is due to cellular proliferation as indicated by increased PCNA counts and an increase in cell number. Both decreased and increased blood flow lead to dedifferentiation of SMCs and apoptosis.

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