Low Blood Flow After Angioplasty Augments Mechanisms of Restenosis

Inward Vessel Remodeling, Cell Migration, and Activity of Genes Regulating Migration

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Abstract—The predominant cause of restenosis after angioplasty is now thought to be inward remodeling, but the mechanisms responsible are unknown. Remodeling in normal vessels is regulated by the endothelium in response to altered shear stress. Although the endothelium is often damaged by angioplasty, restenosis rates after angioplasty have been correlated with impaired coronary flow. Thus, we examined how increases or decreases in blood flow through balloon catheter–injured rat carotid arteries affect vessel morphometry (4, 10, and 28 days), cell migration (4 days), and levels of promigratory mRNAs (2 and 10 days). After 28 days, the luminal area in vessels with low blood flow was significantly less than in those with normal and high blood flow (0.17±0.01 [low] versus 0.24±0.06 [normal] versus 0.30±0.02 [high] mm², P<0.01), predominantly because of accentuated inward remodeling (or reduced area within the external elastic lamina; 0.42±0.02 [low] versus 0.54±0.07 [normal] versus 0.53±0.04 [high] mm², P<0.05). Low flow also enhanced smooth muscle cell migration 4 days after injury by 90% above normal and high flows (P<0.01). Two days after injury, low flow significantly increased levels of mRNAs encoding promigratory peptides (integrin αvβ3, transforming growth factor-β1, CD44v6, MDC9, urokinase plasminogen activator receptor, and β3-inducible gene h3); these changes persisted 10 days after injury and were localized to the neointima. Low blood flow may promote restenosis after angioplasty because of its adverse effect on vessel remodeling, and it is associated with the augmented expression of multiple genes central to cell migration and restenosis. (Arterioscler Thromb Vasc Biol. 2001;21:208-213.)

Key Words: angioplasty ■ restenosis ■ remodeling ■ blood flow

Inward remodeling, or reduction in vessel size, is now considered the predominant cause of restenosis after angioplasty1,2; however, the mechanisms responsible are poorly understood. Mechanical shear stress on endothelial cells, which is due to an alteration in blood flow, is a major regulator of remodeling and vessel size in developing blood vessels3 and in blood vessels affected by atherosclerotic lesions.4 Coronary blood flow is frequently impaired in patients with hypercholesterolemia and advanced age,5 and it can become further impaired after angioplasty,6 raising the possibility that flow may also influence remodeling after angioplasty. In uninjured vessels, endothelial cells are central to structural adaptations to sustained changes in blood flow because of their ability to sense changes in blood flow and to alter production of the growth factors and metalloproteinases required by the vessels to remodel.7 Recent studies indicate that when cultured vascular smooth muscle cells (VSMCs) are in the synthetic phenotype, they respond to shear stress in an analogous manner, altering their production of growth factors.8,9 In the balloon catheter–injured and deendothelialized rat carotid artery, the VSMCs that migrate over the internal elastic lamina to form the neointima rapidly change to the synthetic phenotype, and they maintain phenotypic modulation until at least 2 weeks after injury.10,11 Thus, it is possible that juxtaluminal synthetic VSMCs could respond to abnormal shear forces in a manner similar to the endothelium in uninjured vessels and hence potentially influence inward remodeling. These observations prompted us to examine how reductions in blood flow affect luminal narrowing after experimental angioplasty in the rat carotid artery, through either inward remodeling or enlarging the neointima. Our findings led us to further examine how blood flow affects cellular migration and the molecular events that regulate it.

Methods

Animals and Surgery

Male Sprague-Dawley rats, 400 to 500 g, were anesthetized as previously described,12 and a 2F Fogarty arterial embolectomy catheter (Baxter) was passed through an arteriotomy in the left common femoral into the left common carotid artery to its bifurca-
tion. The balloon was inflated with 25 µL of saline, withdrawn with a rotating action to the aortic arch, and reintroduced, and withdrawn of the inflated balloon was repeated twice. Flow was then reduced by ligating the left internal carotid artery or increased by ligating the right common carotid artery. We and others have previously shown that these ligations reliably decrease blood flow by a mean of 35% and increase flow by 29%, respectively.13,14 Five animals per group were used, and reverse transcription (RT)–polymerase chain reaction (PCR) analysis of whole-vessel mRNA expression was carried out on high- and low-flow vessels 2 days after injury and was compared with that in balloon-injured vessels in which the flow was unaltered after injury. Persistence of these changes was assessed by analysis of mRNA levels in high- and low-flow vessels 10 days after injury. To localize the changes in mRNA expression 10 days after injury within the vessel wall, the arterial layers (intima, media, and adventitia) were separated under a dissecting microscope in an additional 6 high-flow and 6 low-flow injured vessels, and layers from 3 vessels were combined for mRNA analyses (n=2 data points for each layer in each flow group). Animals were euthanized with an overdose of pentobarbital, and vessels were collected for mRNA analyses as previously described.12 Fifteen minutes before perfusion fixation, Evans blue dye (Sigma Chemical Co) was injected intravenously (60 mg/kg) to demarcate the degree of endothelial regrowth in each vessel as previously described.15 Vessels for morphometry were perfusion-fixed with 4% formalin in PBS (pH 7.4) at 90 to 100 mm Hg pressure for 5 minutes, dissected free of surrounding tissue and aortic arch, and reintroduced, and withdrawal pressure was determined, and the assays were validated as previously described15 with the use of a mouse monoclonal anti-human smooth muscle actin antibody (Sigma) and a biotinylated secondary horse anti-mouse antibody (Vector Laboratories). After staining for smooth muscle actin with the use of the chromogen 3,3′-diaminobenzidine tetrachloride and counterstaining with hematoxylin, nuclei present in the neointima 4 days after injury were counted and averaged over the cross sections from the 5 segments of each vessel.

**mRNA Analyses by Standardized RT-PCR**

For analysis of mRNA levels in segments of the injured arteries, total RNA was extracted and treated with DNase (Promega) to ensure a final DNA-free RNA preparation, exactly as previously described.12 Two hundred nanograms of RNA was reverse-transcribed by use of a GeneAmp RNA-PCR kit (Perkin-Elmer), and then specific fragments were amplified from RNAs encoding the disintegrin-metalloprotease MDC9, β1- and β3-integrin, transforming growth factor (TGF)–β2, and its receptors ALK-5 and TβR-II, and the housekeeping gene L7 by use of thermal cycling conditions as previously described.12 The oligonucleotide primers used to amplify the integrins αR, βR, TGF-βR, ALK-5, and TβR-II, and L7 were as previously described.12 The sense and antisense oligonucleotides for MDC9 were targeted to base pairs 1008 to 1037 and 1510 to 1539, respectively, of the rat cDNA; sense and antisense CD44v6 oligonucleotides, to base pairs 691 to 720 and 1111 to 1140, respectively, of the rat cDNA16; sense and antisense β1- and β3-integrin oligonucleotides, to base pairs 680 to 709 and 993 to 1012, respectively, of its rat cDNA;20 sense and antisense uPA oligonucleotides, to base pairs 431 to 460 and 712 to 737, respectively, of its rat cDNA.20 RT-PCR–generated cDNA fragment identities were confirmed by use of either diagnostic restriction endonucleases or nucleotide sequencing (TaQTrack, Promega) after cloning into pGEM-T vectors (Promega). The number of cycles optimum for each primer pair to accurately estimate mRNA levels was determined, and the assays were validated as previously described.12 The amount of PCR product generated from the different oligonucleotide primers was expressed relative to the L7 RT-PCR product, a noninducible cell cycle–independent ribosomal protein that is unaltered during VSMC proliferation.21 PCR products were quantified by laser densitometry from the photographic negatives of agarose gels, in which the fragments were electrophoresed, under UV light. We have previously shown this method to be highly reliable in estimating mRNA levels with R values >0.95 and an average coefficient of variation of <10%.12

**Statistical Analysis**

Results are expressed as mean±SEM. Parameters of vessel structure and changes in mRNA levels were analyzed by ANOVA or ANOVA on ranks as appropriate, followed by multiple pairwise comparison with Student-Newman-Keuls test. A value of P<0.05 was considered statistically significant. Simple linear regression was used to examine the relationship between time and luminal area or vessel area.

**Results**

**Effects of Blood Flow on Vessel Structure After Angioplasty**

Initially, we compared how changes in blood flow through the left common carotid artery made at the time of balloon injury affected its overall structure during healing. Twenty-eight days after injury, the luminal area of the vessels with low blood flow was reduced by 43% compared with those with high blood flow (P<0.01, Figure 1) and by 30% relative to vessels with normal flow (P<0.05, Table 1). The difference in luminal size between arteries with high and low blood flow was progressive with time (Figure 1). Luminal area (LA, mm²) in relation to time (T, days) after injury was best described by the following equations: for low flow, LA=0.569−0.291 log T (R²=0.78, P<0.001); for normal flow, LA=0.575−0.231 log T (R²=0.39, P<0.01); and for high flow, LA=0.581−0.195 log T (R²=0.26, P=0.07).

This was accompanied by a 20% reduction in vessel area, or cross-sectional area within the external elastic lamina (aeEL), compared with vessels with high or normal blood flow (P<0.05). Approximately 80% of the flow-dependent reduction in luminal size was due to reduction in vessel area. The time course of reduction in area encompassed by the external elastic lamina was similar to the time course of luminal loss. In the low-flow vessels, aeEL (mm²) was best described by the following equation: aeEL=0.662−0.168 log T (R²=0.41, P=0.02). The relationships between reductions in vessel area and time were not statistically significant in normal- and high-flow vessels.

The neointima area increased rapidly in the vessels with low blood flow, and 10 days after injury, this area was significantly greater than that in vessels with both high and normal flow. By 28 days, however, this difference was reduced and accounted for only ~20% of the difference in luminal size between high- and low-flow vessels (Table 1).

Neither cross-sectional area nor thickness of the media in the injured vessels was significantly affected by change in blood flow (Table 1). As has been previously described,22 there was no apparent difference between high- and low-flow vessels in
the degree of endothelial regrowth, which was always <25% of the length of the vessel (not shown).

**Blood Flow and Smooth Muscle Cell Migration**

The cellular rearrangements necessary for a vessel to remodel likely involve cellular migration. Others have previously postulated that low flow may augment VSMC migration, inasmuch as low flow does not affect VSMC proliferation rates despite increasing neointimal formation. Augmentation of inward remodeling in vessels with low blood flow appeared to be initiated early after balloon catheter injury, which is consistent with a role for altered cell migration in flow-dependent luminal loss. Although migration is difficult to quantify in vivo, the accumulation of cells in the intima early (∼4 days) after injury is considered to be mostly due to the migration of VSMCs from the injured media. Therefore, we examined how flow affected intimal VSMC accumulation in injured vessels. Four days after the injury, there were 90% more cells in the neointima of vessels with low flow than in those with high and normal flow (P<0.01, Figure 2). Immunohistochemical staining showed that all intimal cells contained α-smooth muscle actin (not shown), confirming their smooth muscle cell identity.

**Blood Flow Regulates Genes Essential for Cell Migration**

We then examined how blood flow influences the activity of genes that others have shown to regulate cell migration: the integrin subunits αv and β3, whose dimer is known to be critical for VSMC migration, and osteopontin (its chief matrix ligand), transforming growth factor-β, and its type I and type II signaling receptors (ALK-5 and TβRII), and β-igh3, which inhibits cell-matrix attachment and is thought to reflect TGF-β bioactivity. Two novel mRNA species were also selected for analysis: the disintegrin-metalloprotease MDC9, which plays a critical role in cell migration through directed membrane-bound protease and Arg-Gly-Asp–blocking activities, and the splice variant of the hyaluronate receptor CD44v6, which is preferentially upregulated after vessel injury and potentiates VSMC migration by increasing interaction with hyaluronate in the extracellular matrix.

Although injury alone (normal flow group) is known to significantly upregulate the expression of these mRNAs, 2 days after balloon injury there was significant further mRNA expression in the low-flow group of MDC9, β-igh3, CD44v6, integrins αv and β3, uPAR, TGF-β1, ALK-5, and TβRII (Table 2). Flow did not affect the expression of osteopontin or urokinase. In vessels with high flow, mRNA levels were similar to those with normal flow. The heightened expression of these mRNA species in low-flow conditions fell over time, although those encoding MDC9, CD44v6, TGF-β1, and integrins αv and β3 continued to be significantly higher 10 days after the initial injury in vessels with low flow compared with those with high flow by 5.7±2.7-, 1.7±0.2-, 3.1±0.9-, 1.7±0.3-, and 1.5±0.3-fold, respectively (all P<0.05). Additional experiments in which RNA was extracted from the neointima, media, and adventitia separately indicated that the higher MDC 9 and CD44v6 mRNA levels in the low-flow
vessels were localized to the neointima but that integrin αv and TGF-β mRNA were greater in the neointima and media of the low-flow vessels than the high-flow vessels (data not shown).

### Discussion

The present study demonstrates that low blood flow in injured deendothelialized arteries accelerates inward remodeling and significantly augments the reduction in luminal size observed late after injury. Reduced flow also potentiates VSMC migration in the injured artery and enhances the mRNA expression of several proteins involved in migration, providing 1 potential contributing mechanism to flow-dependent inward remodeling. These findings have major implications for restenosis after angioplasty, in which the majority of late luminal loss occurs as a result of inward vessel remodeling, because impaired flow is common in atherosclerotic arteries, particularly after angioplasty, and is associated with increased rates of restenosis.

Our findings indicate that injured vessels devoid of endothelium can be remodeled in a flow-dependent manner. Although others have previously used computer modeling of shear stress to show that inward remodeling after angioplasty is correlated with reduced shear, this is the first direct evidence that poor flow in a newly injured vessel will exacerbate inward remodeling. The present study may appear in direct conflict with previous investigators who have found that gentle removal of the endothelium prevents inward remodeling in response to reduced flow. However, in these studies, the atraumatic techniques used for gentle deendothelialization appeared not to induce the usual injury-induced medial smooth muscle cell phenotypic modulation (from “contractile” to “synthetic”), inasmuch as neointimal formation due to cell migration and proliferation was absent and vasoconstriction to neoepinephrine was preserved. Phenotypic modulation may be critical in determining the response to altered flow because flow-responsive production of the growth factors and matrix metalloproteinases to which flow-sensitive remodeling has specific relevance to restenosis after coronary angioplasty, in which flow-exposed VSMCs are also dedifferenti-
ated for some time after human percutaneous transluminal coronary angioplasty.35,36

Although the most likely causes of the effects of flow on remodeling, migration, and mRNA expression are shear-responsive transcriptional events, other factors, including the effects of flow on platelet activation, paracrine cell activation, vessel wall stretch, and compression due to wave-form changes, may play a role. The minor endothelial regrowth observed in high- and low-flow vessels suggests that other flow-exposed cells are responsible for the flow-dependent remodeling. In the present study, flow-dependent mRNA expression 10 days after injury was chiefly localized to the neointima, which is consistent with production by flow-exposed synthetic smooth muscle cells.

Remodeling is a complex and poorly understood process that involves alteration in the balance between cell proliferation and apoptosis31 and matrix protein production and degradation.32,33 Augmented smooth muscle cell migration (within the media, from the media to the intima or adventitia, and vice versa) likely also contributes to the structural rearrangement of vascular remodeling. In the present study, increased VSMC migration into the intima was associated with accentuated inward remodeling and may be a surrogate marker for total vessel cellular motility. Such morphological measures of stimulated migration were preceded by enhanced expression of mRNAs, which have previously been reported to be important in cell migration. Several of these mRNAs and their immunoreactive protein products are significantly upregulated by injury itself23,29,32 and have been implicated in negative remodeling32,33 and restenosis after angioplasty.26,40 However, although the genes all affect cell migration, they also have significant effects on cell proliferation/apoptosis (TGF-β1 and integrin α5β3) and matrix turnover (TGF-β1 and uPA/αuPAR), and their association with enhanced inward remodeling may reflect their influence on multiple components of the remodeling process. Intervention to specifically block either cell migration or these proteins or both would enhance our appreciation of the relationship between these genes, cell migration, and remodeling.

In the present study, low flow significantly enhanced neointimal formation at 10 days, but this effect declined and was not significant at 28 days, similar to the pattern of growth in previous studies.13 The lack of growth of the intima in low-flow vessels between 10 and 28 days likely reflects the stimulatory effect of low flow on apoptosis,28 which becomes increasingly important in this time period,41 or the normalization of shear stress resulting from luminal loss and inward remodeling.

Interestingly, there were no differences in migration, migratory protein expression, and remodeling between high- and normal-flow vessels. Previous studies of flow-mediated outward remodeling in uninjured vessels have demonstrated no vessel enlargement with up to 60% increases in flow, although much larger increases stimulate vessel enlargement.42 The authors concluded that the events that regulate shear-responsive outward remodeling may have a threshold for activation, and it appears that similar thresholds may exist in injured vessels.

The findings of the present study have specific relevance for restenosis after human angioplasty and stenting. Impaired coronary flow may persist after dilatation of flow-limiting stenoses because of microvascular dysfunction, infarction of some of the tissue downstream, or the presence of persistent collateral circulation. An excess incidence of restenosis is observed when impaired coronary flow reserve persists after angiographically successful angioplasty,29,30 and it is observed specifically in patients with persistent collateral flow after the opening of a chronic total occlusion.43 Although inward remodeling does not occur in stented vessels, low flow also appears to increase stent restenosis44; this increase is presumably due to prolonged stimulation of intimal hyperplasia by persistently low shear stress in the absence of inward remodeling.

In conclusion, the present study indicates that blood flow is an important regulator of vessel remodeling after angioplasty and that reduced blood flow enhances many mechanisms involved in cell migration, providing a link between the effects of flow on molecular, cellular, and morphological processes. Although the basic molecular mechanisms by which low flow ultimately affects gene activity remain to be fully elucidated, these responses provide potential targets through which it may be possible to influence remodeling after angioplasty.

Acknowledgments

This work was funded in part by a NH&MRC of Australia block grant and an NHF of Australia project grant. Michael Ward was supported by an NH&MRC (Australia) postgraduate medical scholarship.

References

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doi: 10.1161/01.ATV.21.2.208

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