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ORIGINAL ARTICLE

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Ultrastructure and molecular histology of rabbit hind-limb collateral artery growth (arteriogenesis)

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Abstract Previous studies in the canine heart had shown that the growth of collateral arteries occurs via proliferative enlargement of pre-existing arteriolar connections (arteriogenesis). In the present study, we investigated the ultrastructure and molecular histology of growing and remodeling collateral arteries that develop after femoral artery occlusion in rabbits as a function of time from 2 h to 240 days after occlusion. Pre-existent arteriolar collaterals had a diameter of about 50 µm. They consisted of one to two layers of smooth muscle cells (SMCs) and were morphologically indistinguishable from normal arterioles. The stages of arteriogenesis consisted of arteriolar thinning, followed by transformation of SMCs from the contractile- into the proliferative- and synthetic phenotype. Endothelial cells (ECs) and SMCs proliferated, and SMCs migrated and formed a neo-intima. Intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) showed early upregulation in ECs, which was accompanied by accumulation of blood-derived macrophages. Mitosis of ECs and SMCs started about 24 h after occlusion, whereas adhesion molecule expression and monocyte adhesion occurred as early as 12 h after occlusion, suggesting a role of monocytes in vascular cell proliferation. Treatment of rabbits with the pro-inflammatory cytokine MCP-1 increased monocyte adhesion and accelerated vascular remodeling. In vitro shear-stress experiments in cultured ECs revealed an increased phosphorylation of the focal contacts after 30 min and induction of ICAM-1 and VCAM-1 expression between 2 h and 6 h after shear onset, suggesting that shear stress may be the initiating event. We conclude that the process of arteriogenesis, which leads to the positive remodeling of an arteriole into an artery up to 12 times its original size, can be modified by modulators of inflammation.

Key words Arteriogenesis · Collateral vessels · Ultrastructure · Cell adhesion molecules

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Introduction

Vascular growth proceeds via three distinct mechanisms: *angiogenesis* is the sprouting or de novo growth of capillaries; *vasculogenesis* is the in situ development of vessels from angioblasts in the early ontogenesis; and *arteriogenesis* is the growth of collateral arteries from pre-existing arterioles in adults [6, 21, 32, 38]. Previous studies indicated that these processes are differently regulated. Vascular endothelial growth factor (VEGF), an endothelial-specific mitogen, has been shown to induce developmental and tumor angiogenesis [7, 8], especially under hypoxic/ischemic conditions. However, when we studied collateral growth in the rabbit hind limb, we were not able to detect any perfusion deficits or biochemical indicators of ischemia in regions of collateral artery growth [27]. For these reasons, it is unlikely that collateral arteries grow via angiogenesis. It is more likely that these vessels grow from pre-existing arteriolar anastomoses, which have been described in the canine as well as in the human heart [5, 22, 37]. These interconnecting arterioles respond to pressure differentials that develop in response to stenoses and occlusions, which increase directional flow and hence shear stress. The development of pre-existing canine coronary arterioles into small arteries was described in ultrastructural detail by our group years ago [36, 37]. The present study was carried out to reveal whether rabbit hind-limb collaterals follow the same stages as canine coronary collaterals. Our previous findings of monocyte involve-

ment in arteriogenesis in the canine heart [35] as well as in the rabbit hind limb were the basis of our monocyte chemoattractant protein (MCP-1) infusion studies, the morphological consequences of which are described here. Since monocytes cannot adhere to endothelium without prior upregulation of adhesion molecules, we also studied their kinetic expression following femoral occlusion.

Material and methods

Animal model

The present study was performed with permission of the State of Hessen, Regierungspräsidium Darmstadt, according to section 8 of the German *Law for the protection of animals*. It conforms with the *Guide for the Care and Use of Laboratory Animals* [31]. In New Zealand white rabbits, the right femoral artery was occluded as previously described [27]. Briefly, animals were anesthetized with 50 mg/kg ketamine and 5 mg/kg xylasin, and the femoral artery was occluded with two knots; then, the skin was closed and the animals were allowed to recover. After 2, 4, 8, 12, and 24 h, and 2, 3, 5, 7, 14, 21, 42, 180 and 240 days of occlusion, rabbits were euthanized with an anesthetic overdose, and the abdominal aorta was incised, cannulated and perfused with a bismuth–gelatin-based contrast medium or with 2% formaldehyde (see below).

MCP administration

Four rabbits received MCP-1 (PeproTechInc.) locally via an osmotic minipump (2ML-2, Alza Corp); 3 mg in 2 ml phosphate-buffered saline (PBS) at a rate of 10 µl/h for 7 days.

Perfusion fixation and tissue sampling

Hind limbs were perfused as described previously [27]. Briefly, first the rinsing solution [PBS containing 5 mM ethylene diamine tetraacetic acid (EDTA), 0.5% bovine serum albumin (BSA) (Fluka) and 0.3 mg/1 adenosine] was infused via the abdominal aorta under a hydrostatic pressure of 90 cm water for 2 min, followed by 2% formaldehyde in the same buffer, but without BSA, for 15 min. White barium-based contrast medium was injected at 37°C, as described [27], to make the collateral vessels visible for sampling. Large collateral arteries were sampled from the red portion of the quadriceps muscle adjacent to the thigh bone; this site was chosen to guarantee consistency for the follow-up morphological study. Collaterals that had developed in the adductor muscle were also included in the study.

Ultrastructural study

For electron microscopy, rabbit tissue was perfusion fixed and filled with bismuth–gelatin contrast to mark vessels; these were then dissected, cut in 2- to 3-mm sections, post-fixed for 4 h in 4% glutaraldehyde, followed by 4 h in OSO_4. After dehydration in graded alcohol, samples were embedded in Epon and sectioned transversally. Semi-thin sections were stained with toluidine blue and photographed with a Leica DM microscope (Leica, Bensheim, Germany). Semi-thin sections were also used for the measurement of collateral diameters. Ultra-thin sections were stained with uranyl acetate and lead citrate, viewed and photographically recorded using a Philips CM 10 electron microscope. Ultrastructural changes in at least three collateral vessels from a minimum of two animals per time point (2, 3, 6, 7, 10, 14, 21, 42, 180, and 240 days) were investigated. Vessels from the non-operated left hind limb served as the control.

Immunofluorescence

Specimens were snap-frozen in methylbutane at –130°C and stored at –80°C. Perfusion-fixed tissue was cryoprotected with 20% sucrose before freezing. Cryosections 10- to 20-µm thick were prepared with a Leica CM 3000 Cryomicrotome (Germany), mounted onto silicone-coated slides and incubated overnight in primary antibodies (see below), followed by biotinylated secondary antibody (Dianova, Hamburg, Germany) and Cy2-Streptavidin (Rockland). Finally, nuclei were stained with 0.01% 7-actinomycin D (7-AAD, Molecular Probes). A monoclonal antibody against phosphotyrosine (PY20, Transduction Laboratories, Calif.) was used to localize phosphorylated proteins in situ. Monoclonal antibodies against rabbit intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) were kindly supplied by Dr. M.I. Cybulsky [45], Harvard Medical School, Boston, Mass. Anti-human antibodies were commercial E-selectin, ICAM-1 (clone 15.2, Leinco), and human PECAM-1 (clone JC/70, Dako). Monocytes/macrophages (blood-derived monocytes) were labeled using the human macrophage-associated antigen CD68 (DAKO), described to bind to rabbit macrophages [2], or rabbit macrophage-associated antigen RAM-11 [24]. Cryosections of the rabbit spleen were used as a positive control. The monoclonal antibody MIB-5 (Dianova, Hamburg, Germany) reacts with the nuclear protein Ki-67, which is present in all active phases of the cell cycle and is a reliable marker of proliferation. We used the original method of boiling tissue sections in citrate buffer [12], adapting it for immunofluorescence. Intestinal tissue was used as a positive control.

Quantification of growth

All measurements were performed on semi-thin cross-sections after perfusion fixation under maximal vasodilatation with adenosine. Black and white prints were scanned using the HP ScanJet 4C scanner and Adobe PhotoShop 5.0 software. The diameter of the vessel lumen was calculated using the National Institutes of Health (NIH) Image 1.62 program.

RNA isolation and Northern hybridization

Total RNA was isolated from frozen tissue of the quadriceps muscle containing collateral vessels according to the method of Chomzynski and Sacchi [16], and Northern hybridization was carried out according to standard procedures [34]. Fifteen micrograms of total RNA from control, sham-operated rabbits or experimental tissue were size fractionated per slot on a 1% agarose gel containing 0.66 M formaldehyde. The integrity of the RNA was judged under ultraviolet light. After capillary transfer to a Hybond-N^+ membrane (Amersham) using 10×sodium saline citrate (SSC; 1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) as transfer buffer, the RNA was fixed to the filter by ultraviolet crosslinker (Stratagene). For Northern-blot analysis, cDNA probes were random prime labeled to a specific activity of about 10^8 cpm/mg using a *redi*prime labeling system (Amersham) and 40 mCi of (α-^{32}P) dCTP (3000 Ci/mmol). cDNA probes used in our studies were as follows: VCAM-1 (rabbit, 700 bp, kindly provided by M.I. Cybulski), ICAM-1 (mouse, 2520 bp, ATCC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; human, 720 bp, ATCC), and 18s (mouse, 770 bp, kindly provided by I. Oberbäumer). Northern blots were hybridized with a probe specific for 18s rRNA to confirm equal loading.

In vitro shear stress

Primary cultures of human umbilical vein endothelial cells (HUVECs) were grown to confluence and then deprived of serum for 24 h. We chose HUVECs because of their availability and because they were recently shown to answer to shear stress similar to

arterial endothelial cells [20]. Cells were then mounted in a cone/plate viscosimeter and exposed to a constant fluid shear stress (3 dyn/cm^2 or 12 dyn/cm^2) for 10 min, 20 min, 30 min, 2 h or 6 h. Thereafter, cells were rapidly fixed in 4% buffered formaldehyde. Tumor necrosis factor (TNF)-α (100 U/ml, 90 min) was administered for the induced expression of the adhesion molecules and served as positive control [40].

In vitro proliferation assay

To determine the time course of endothelial cell reaction to growth factor stimulation, in vitro experiments were performed using cultured bovine aortic endothelial cells. Confluent endothelial cells were deprived of serum for 24 h prior to the addition of basic fibroblast growth factor (bFGF, 10 ng/ml). The cells were fixed 2, 4, 6, 8, 10, 18, and 24 h thereafter, and labeled with antibodies against K_i – as described for the tissue sections, but using a peroxidase–diaminobenzidine (DAB)–H_2O_2 reaction. The percentage of positive cells was counted using a Leica DM microscope.

Results

Ultrastructure and histology

Two to three days after occlusion of the femoral artery, endothelial cells and about a half of the SMCs contained many free ribosomes and lesser amounts of rough endoplasmic reticulum (Fig. 1A), suggesting that these cells exhibited proliferative rather than synthetic activity. The lamina elastica interna had partially disappeared (Fig. 6A). In a number of vessels, a double elastic lamina indicated the beginning of neo-intima formation. All these alterations were localized in the midzone of the growing vessel. Proximal (stem) as well as distal (re-entry) parts showed normal structure at this time point, beginning the remodeling a little later. Five to seven days after occlusion, a very prominent rough endoplasmic reticulum suggested strong synthetic activity in all layers of the arterial wall (Fig. 1B). Rare monocytes were vacuolized and degenerating (not shown). In contrast, a plethora of monocytes was detected 7 days after occlusion around the collateral arteries in animals treated with MCP-1.

Ten to fourteen days after femoral occlusion, a neo-intima was evident. The proximal layer of smooth-muscle cells (SMCs) still retained the synthetic phenotype, whereas other layers had reverted to the contractile phenotype (Fig. 1C). The abundance of the rough endoplasmic reticulum in the endothelial cells and adventitial fibroblasts suggested significant synthetic activity. The extracellular matrix (ECM) contained cellular debris. The compact parts of the original internal elastic lamina were connected with the less compact newly synthesized elastic material. Twenty-one days after occlusion, the asymmetric neo-intima contained maximally three to four layers of SMCs (Fig. 1D) and, at 42 days after occlusion, six to seven layers with the new elastic material between each two layers (Fig. 1D and Fig. 2A, B). The majority of SMCs in the neo-intima as well as all SMCs in the media belonged to the contractile phenotype. Medial SMCs were directed circularly to the vessel axis as in normal vessels, whereas the intimal SMCs were smaller, helically directed and contracted (Fig. 2).

The neo-intima in the stem and the re-entry parts of collaterals were much thinner: only one to two layers of SMCs. In collaterals from the adductor region, the neo-intima was generally not as prominent as in the quadriceps. In some cases, the neo-intima was asymmetric, half-moon-like or showed foci of sub-endothelial growth under which the lamina elastica interna was disrupted and the media disorganized. These sites probably represent the point of immigration of the medial SMC into the neo-intima. Elastic material of the former lamina elastica interna could be easily differentiated from the newly synthesized because of its thickness; it labeled the border between the media and the neo-intima. This new elastic material represented a significant portion of the increased neo-intimal ECM. Many collagen fibers were present in the adventitia. One-hundred and eighty to two-hundred forty days after occlusion, the ultrastructure of collateral vessels was similar to that after 21–42 days (Fig. 3). All SMCs belonged to the synthetic phenotype; endothelial cells were quiescent. The neo-intima consisted of one to four layers of SMCs. Complete normalization of structure was not observed, even after long periods of observation.

Quantification of growth

Pre-existing collateral arterioles are morphologically indistinguishable from normal vessels; we could be sure to deal with collateral vessels only after they had become visible. Therefore, we measured the vascular diameter beginning from the second day after the occlusion. Results of independent measurements of at least two arterial vessels from each hind limb of at least two rabbits per time point are presented in Fig. 4. It is obvious that the growth was maximal during the first 3 weeks and almost complete at 21 days after occlusion, although a low proliferative activity can be observed even after 21 days (Fig. 9). The MCP-1 treatment does not change vascular diameters (not shown).

Monocytes/macrophages assemble around the growing collaterals

We used a CD-68 antibody against human monocytes/macrophages known to cross-react with rabbit tissue. Vessels in the control skeletal muscle tissue taken from the non-operated leg as well as from the operated side 2, 4, or 8 h after femoral artery occlusion expressed no CD68. The first CD68-positive cells were observed 12 h after occlusion (Fig. 5). The maximal number of macrophages was observed 2–3 days after occlusion, i.e., the time point when they were also found morphologically on semi-thin sections (Fig. 6). Monocytes appeared in clusters, mostly in the midzone of collateral vessels within an area of less than 2 mm of vessel length.

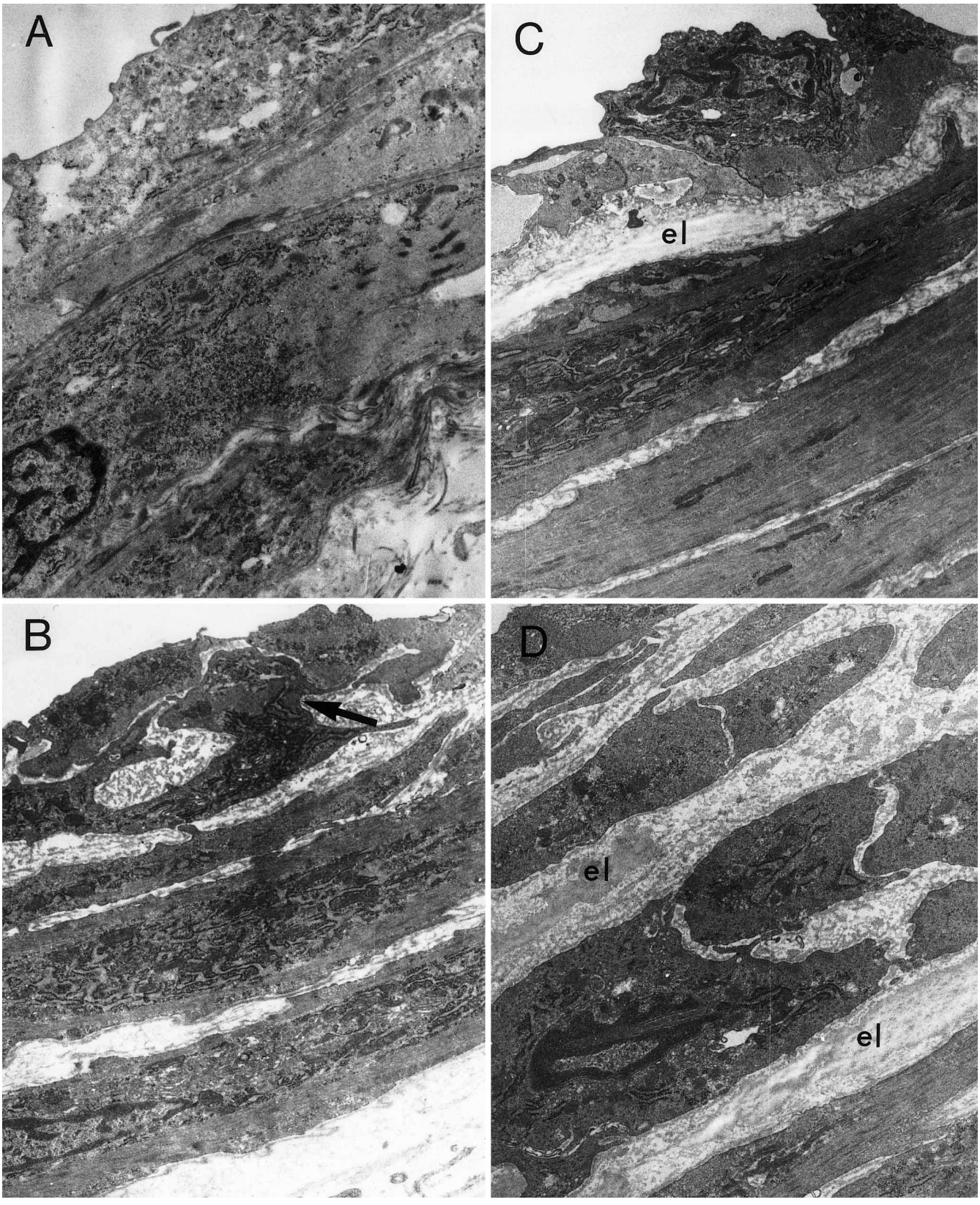


Fig. 1 **A** Ultrastructure of the collateral vessel wall 2 days after the femoral artery occlusion. Smooth muscle cells (SMCs) ("proliferative" phenotype) contain numerous free ribosomes (×8800). **B** Ultrastructure of the collateral vessel wall 5 days after femoral artery occlusion. SMCs ("synthetic" phenotype) contain much endoplasmic reticulum. One SMC (*arrow*) seems to move in the direction of the lumen; the lamina elastica interna is not distinguishable (×8400). **C** Ultrastructure of the collateral vessel wall 14 days after femoral artery occlusion. SMCs return to the contractile phenotype, only first (from the lumen) layer remains synthetic. El-elastic membrane (×8400). **D** Ultrastructure of the collateral vessel wall 21 days after the femoral artery occlusion. Double lamina elastica interna (el), enlarged space between SMCs contains extracellular matrix (×8400)

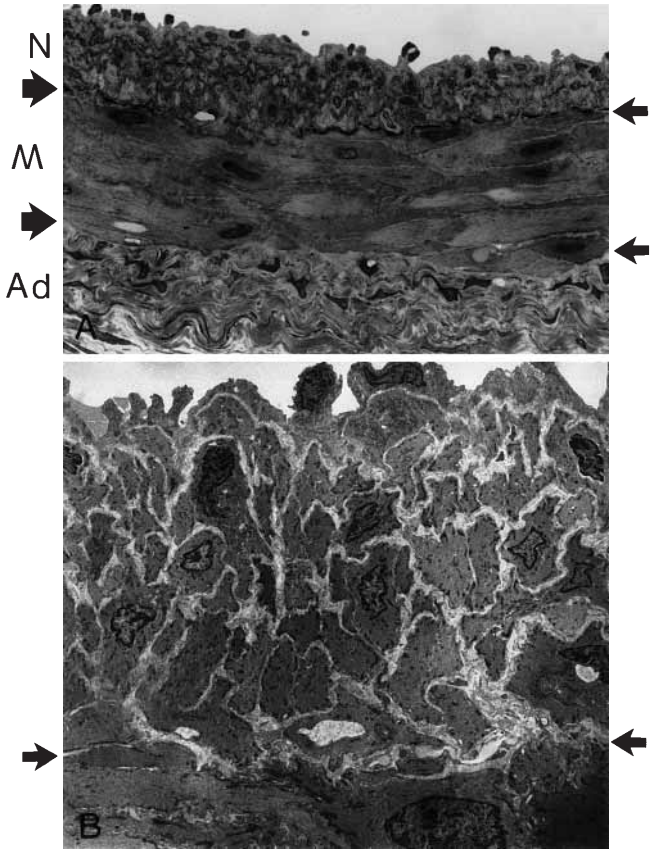


Fig. 2 Ultrastructure of the collateral vessel wall 42 days after femoral artery occlusion. **A** Semi-thin section. Ruptured thick *black line* (labeled from left and right with *thick arrows*) is the old lamina elastica interna; it marks the bottom of the neo-intima (*N*). Smooth muscle cells (SMCs) in the neo-intima are smaller than those in the media (*M*). The adventitia (*Ad*) containing collagen and the lamina elastica externa appears normal (×800). **B** Ultra-thin section. All SMCs are contractile; those of the neo-intima are smaller than in the media and differently directed. Remnants of the old lamina elastica interna labeled with *arrowheads* (×3000)

Endothelial adhesion molecules

We analyzed the mRNA expression of ICAM-1 and VCAM-1 during the first 24 h (i.e., at 3, 6, 12, and 24 h) after femoral occlusion in the quadriceps region. Neither transcript could be detected in control or sham-operated animals. However, we found a signal for ICAM-1 at 12 h and 24 h after occlusion (Fig. 7A) and for VCAM-1 at 12 h (Fig. 7B) after occlusion. For VCAM-1, we found two mRNA species, in agreement with former data [19, 29]. At the protein level (immunohistochemically), the control vessels and those until 2–8 h after ligation showed no positive staining for VCAM-1. Venules and veins, but not arteries, arterioles or capillaries, expressed ICAM-1 constitutively (Fig. 8A). In control vessels, no VCAM-1 labeling was detected. The positive reaction for both ICAM-1 and VCAM-1 was found in endothelial and to a lesser extent in SM cells between 12 h (Fig. 8B) and 48 h (Fig. 8C) after femoral occlusion. Similar timing was found for the VCAM-1 expression (Fig. 8D). No

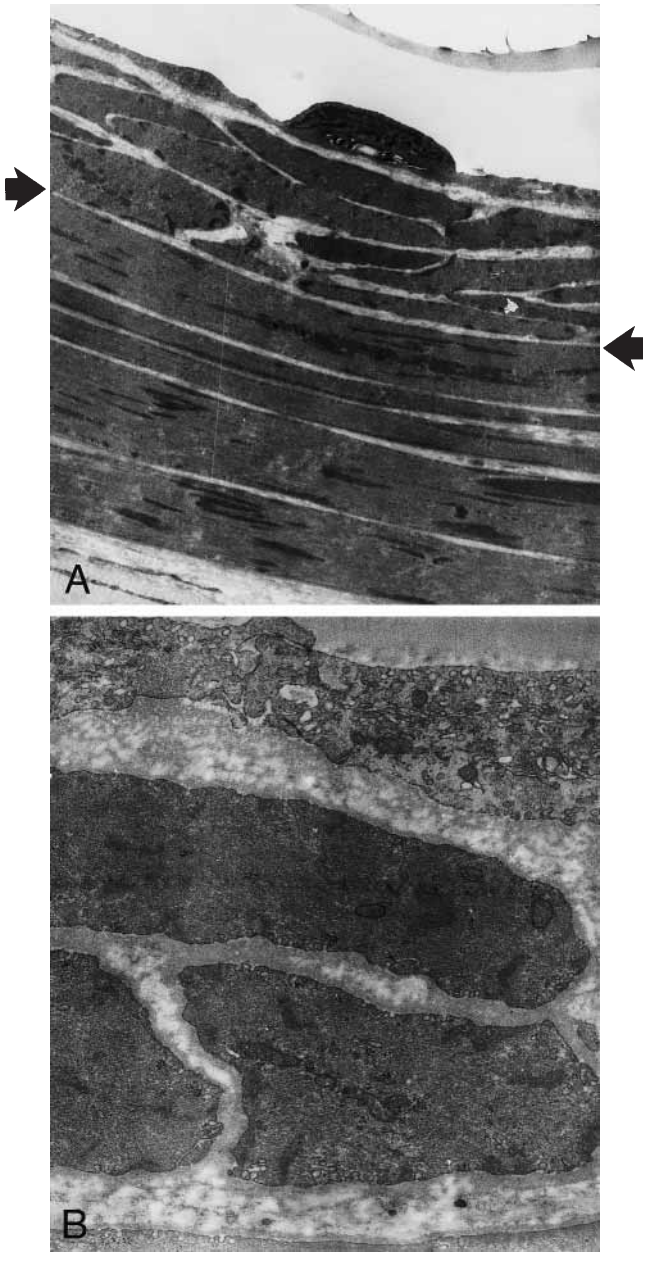


Fig. 3 **A** Ultrastructure of the collateral vessel wall, 240 days after femoral artery occlusion. The neo-intima–media border is labeled with *arrows*. **B** At larger magnification, elastic material is prominent between endothelial cells and smooth muscle cells (SMCs), but also between two layers of SMCs, and even between neighboring SMCs at the same layer. **A** ×8200; **B** ×23,000

ICAM-1 or VCAM-1 signals were detected later than 3 days after occlusion. Only a small portion of arterioles showed positive staining for ICAM-1 and VCAM-1, suggesting that only few collateral vessels start to grow.

Proliferation

The first positive Ki-67 reaction in the vessel wall of growing collaterals was found 24 h after occlusion in the

midzone of collaterals from the quadriceps region. The reaction was stronger after 2 days (Fig. 9A), increased further by day 3 (Fig. 9B) and extended from the midzone to proximal (stem) and distal (re-entry) regions. In the calf region, the Ki-67 positive reaction was found in capillaries, but never in arterioles (not shown), which suggests that angiogenesis rather than arteriogenesis had taken place. A diminished Ki-67 reaction was found in collateral arteries at 7 days (Fig. 9C) as well as 21 days (Fig. 9D) and still at 42 days, but disappeared after 180 days (not shown). To test the time course of proliferation under the influence of growth factors, bovine aortic endothelial cells in culture were studied. Unstimulated cells were stained for Ki-67 at a rate of 6%. The percentage of Ki-67-positive cells and the labeling intensity increased to 13–30% in the course of 8–24 h after the addition of bFGF (results not shown).

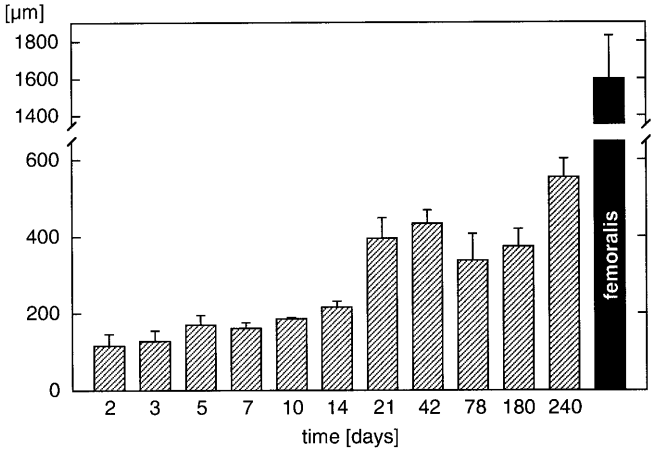


Fig. 4 Measurements of the diameter of growing collateral arteries compared with the femoral artery

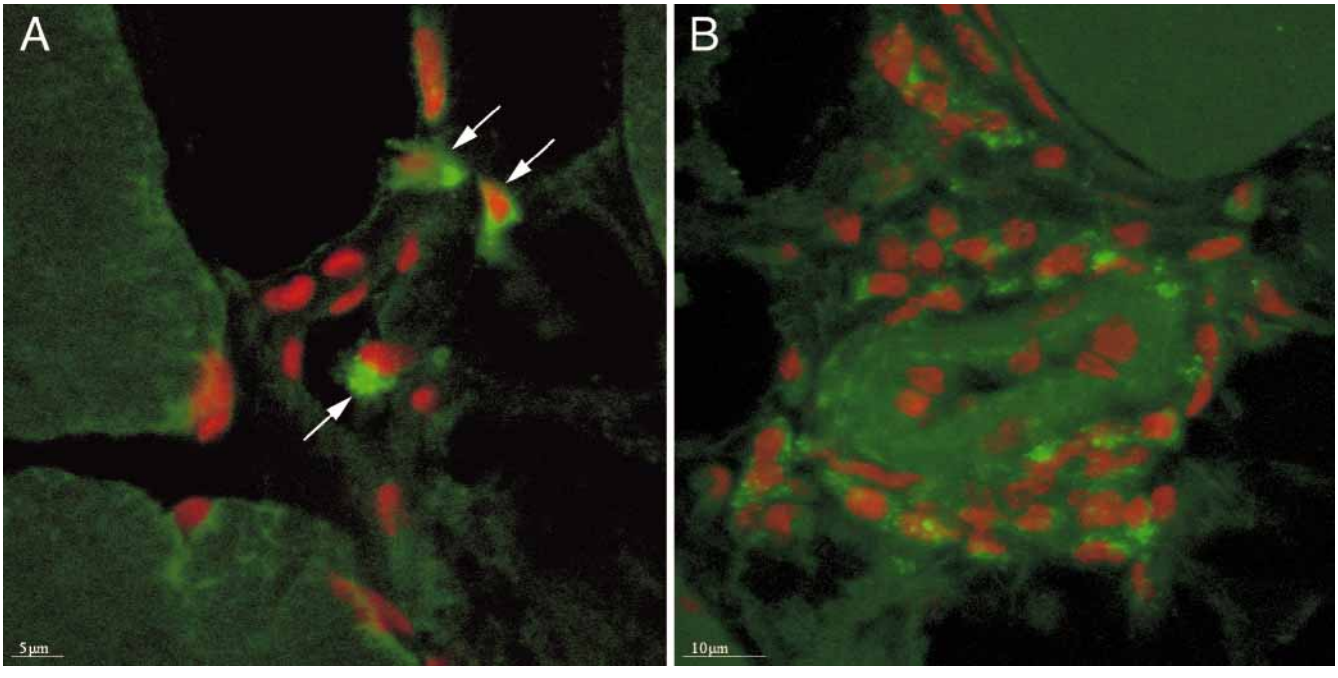
Fig. 5 **A** Collateral vessel with one CD-positive cell in lumen adhering to the endothelial cell and two CD-positive cells (*arrows*) in the adventitia 12 h after occlusion of the femoral artery. **B** Many CD-68-positive macrophages 16 h after occlusion

Adhesion molecules are upregulated by fluid shear stress

In unstimulated primary cultures of HUVECs, ICAM-1 was constitutively expressed, but no signal for either E-selectin or VCAM-1 could be detected, as we already described [40]. (Fig. 10A–C). TNF-α (100 U/ml, 90 min; Fig. 10D–F) was used as a positive control to induce adhesion molecule expression, although exposure to the cytokine was not long enough to induce the expression of VCAM-1, as described previously [40]. Two to six hours of shear stress of 12 dyn/cm^2 induced E-selectin, ICAM-1, and VCAM-1 perinuclearly, suggesting newly synthesized protein (Fig. 10G–L); a weaker shear stress of 3 dyn/cm^2 failed to induce the adhesion molecules (not shown). Newly synthesized protein was localized perinuclearly following TNF-α stimulation, but was more diffuse, probably cytoskeleton-associated, following exposure to fluid shear stress.

Tyrosine phosphorylation in endothelial cells is modified by fluid shear stress

To investigate the signaling processes activated by the application of fluid shear stress to endothelial cells, we studied changes in cellular tyrosine phosphorylation in HUVECs exposed to shear stress (12 dyn/cm^2) between 10 min and 14 h. Under basal conditions, phosphotyrosine staining was punctate in nature and corresponded to



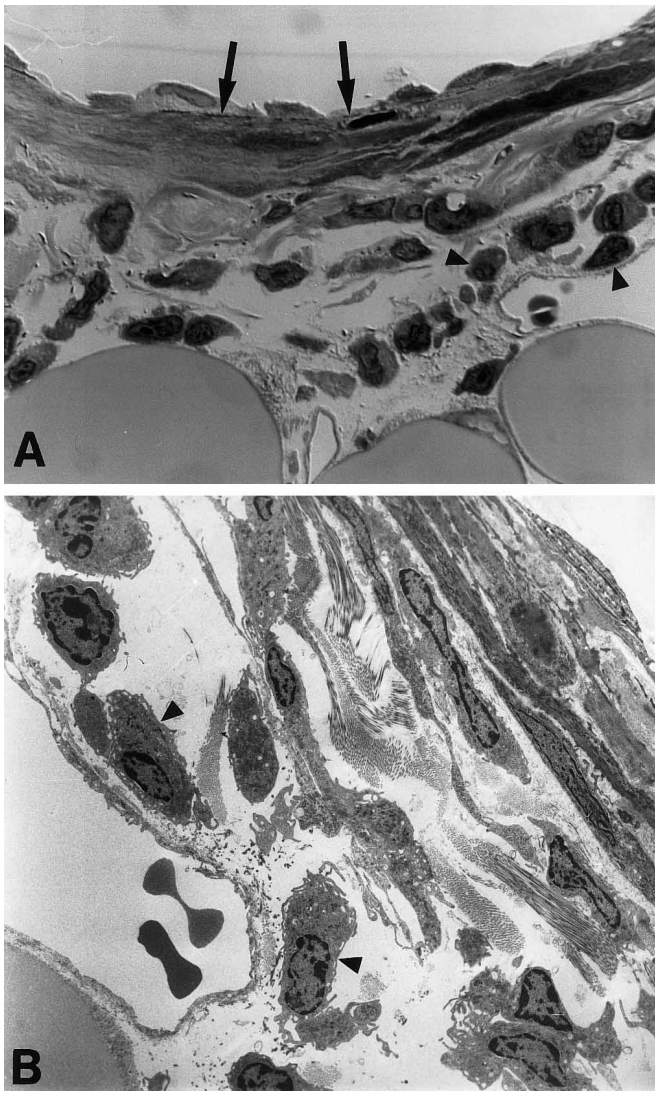


Fig. 6 Perivascular accumulation of macrophages (*arrowheads* label same cells on **A** and **B**), the lamina elastica interna is only partially preserved (*arrows*) 2 days after occlusion of the femoral artery. **A** Light microscopy; semi-thin section, stained with toluidine blue (×1250). **B** Electron microscopy of the same sample; ultra-thin section, contrasted with uranil acetate and lead citrate (×2800)

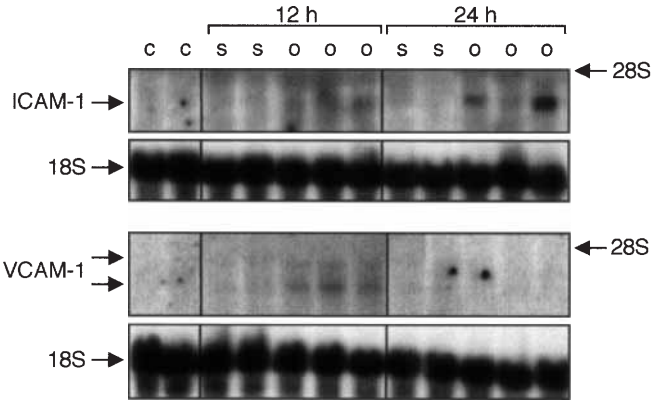


Fig. 7 Northern-blot analysis of intercellular adhesion molecule (ICAM-1) (A) and vascular cell adhesion molecule (VCAM-1) (B). Total RNA isolated from muscle samples (quadriceps) of control (*c*) rabbits, sham-operated rabbits (*s*) and from rabbits 12 h and 24 h after occlusion of the femoral artery (*0*). ICAM-1 is absent in "control" and "sham", but present in "operated" at 12 h and 24 h. VCAM-1 is only visible at 12 h in "operated"

focal adhesion contacts and endothelial cell-to-cell contacts. Prominent changes in the phosphorylation pattern were observed 30 min after the onset of shear stress, with focal adhesion contacts staining more intensely and cell-to-cell contacts weaker. After 14 h of shear stress, only cell-to-cell contacts were clearly phosphorylated (Fig. 11).

Discussion

We describe here, for the first time, the ultrastructure and the molecular histology and biology of the development of a collateral circulation in the rabbit hind limb as a function of time after acute ligation of the femoral artery. Our main findings are:

1. Ultrastructural activation of endothelial cells and upregulated adhesion molecule expression (by immune fluorescence and on the mRNA level) is the first sign of positive remodeling in pre-existent arterioles after acute femoral artery occlusion.
2. Monocytes/macrophages adhere to the endothelium and also appear in the adventitia of growing collaterals before mitotic vascular cells are detectable. MCP-1 infusion increased number and residence time of monocyte clusters around growing collaterals.
3. A prime trigger of adhesion molecule upregulation is increased shear stress which we studied in vitro.
4. Pre-existent arterioles mature into arteries after increasing their internal diameter by a factor of four to five times. Some of these vessels differ morphologically from normal arteries by the persistence of a neointima.

These findings, together with our previous results, enabled us to formulate a hypothesis, by which acute femoral artery occlusion leads to an abrupt increase of fluid shear stress along the shortest track within the interconnecting network of arterioles at the interface with a normally perfused region, caused by the fall in perfusion pressure and, hence, the rise in the pressure difference between adjacent perfusion areas. Shear stress is known to increase the expression of a number of genes including adhesion molecules and endothelial MCP-1 [10, 28, 42], a potent chemoattractant for monocytes. Our finding of early upregulation of ICAM-1 and other adhesion molecules as well as the subsequent attachment of monocytes to the endothelium and the invasion of the adventitia of remodeling arterioles by monocytes (through the wall of the collateral arteriole or adjacent venule; this question needs further investigation) are the basis of our hypothe-

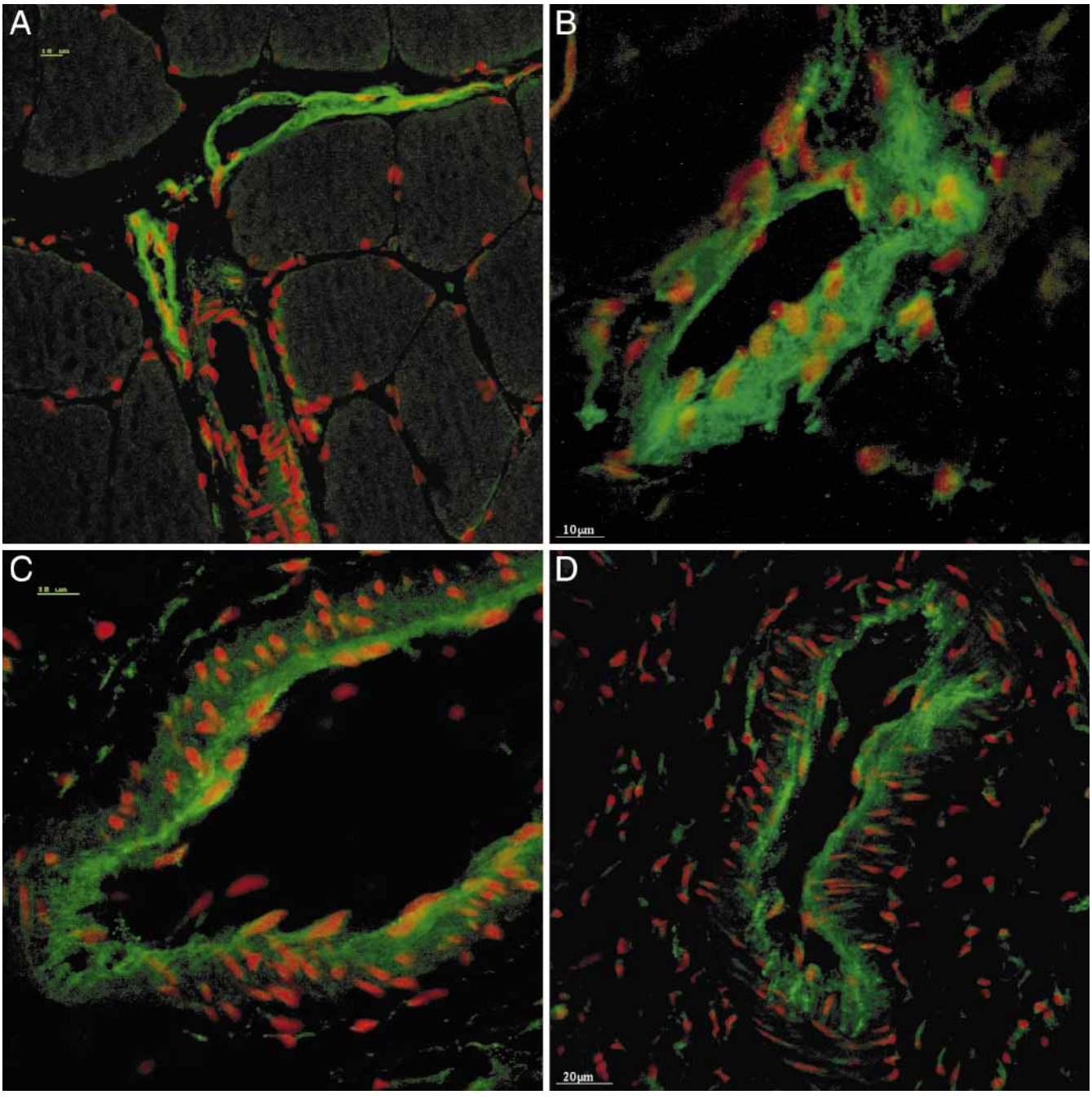


Fig. 8 Expression of adhesion molecules in growing collateral vessels. **A** Intercellular adhesion molecule (ICAM-1) in control tissue. Veins are positive, arteries and capillaries negative. **B** ICAM-1 after 12 h occlusion. Endothelial and smooth muscle cells and some fibroblasts in the arteriole are positive. **C** ICAM-1 after 48 h occlusion. The labeling is weaker but more homogeneous than at 12 h. **D** Vascular cell adhesion molecule (VCAM-1) after 48 h occlusion. Endothelial cells are strongly labeled

sis, which was also experimentally tested by the study of cultured cells subjected to shear stress and which showed expression patterns similar to those observed in vivo. Some of our other main findings, i.e., the upregulation of adhesion molecules and the accumulation of monocytes, were also experimentally tested for their relevance by the application of antibodies against ICAM-1 and by the stimulation of monocytes by MCP-1. We show that collateral artery formation (arteriogenesis) proceeds in an environment of inflammation and that modulation of inflammation modulates also arteriogenesis.

Time course

The stages of arteriogenesis can be classified into initiation, proliferation, synthesis/migration and maturation (Fig. 12). The *initial* phase begins minutes after the femoral occlusion with the activation of the endothelial cells

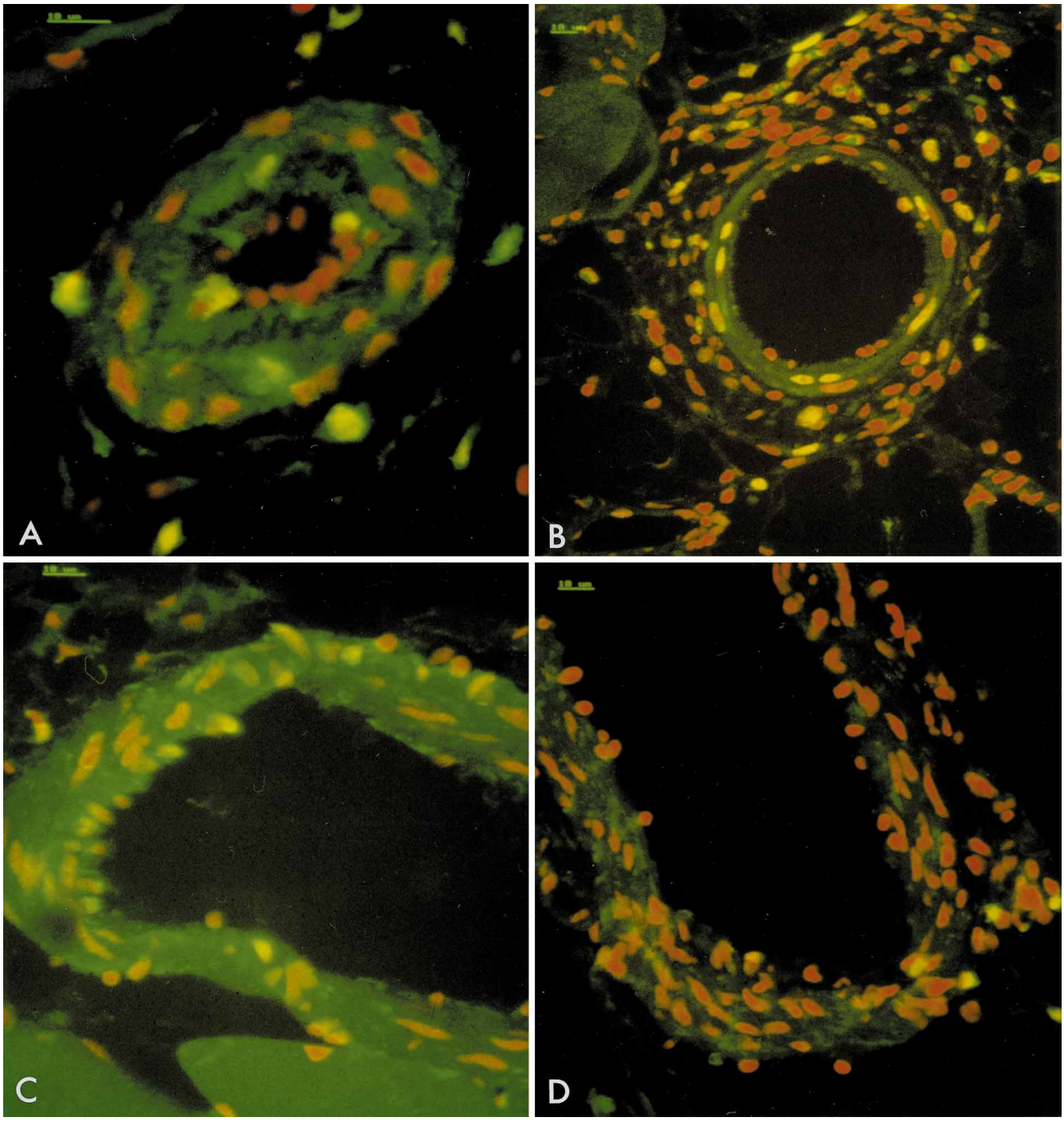


Fig. 9 Ki-67 expression in the wall of a growing collateral artery 3 days after occlusion of the femoral artery (*arrows*). **A** 2 days after occlusion (×1000). **B** 3 days after occlusion (×400). **C** 7 days after occlusion (×670). **D** 21 days after occlusion (×570)

by shear stress and is followed hours later by the induction and/or upregulation of adhesion molecules, and the subsequent adhesion of blood monocytes. The *proliferative* phase (1–3 days after occlusion) is characterized by a maximal mitotic activity in the endothelial cells, SMCs and fibroblasts. The *synthetic/growth* phase (3–14 days) is characterized by the most significant growth, downregulation of the mitotic but initiation of synthetic and proteolytic activity in SMCs, that is reflected in their ultrastructure. The lamina elastica interna, the main barrier to SMC migration, is digested and fragmented by the elastolytic enzymes facilitating positive remodeling. At this phase, monocyte recruitment had ceased and adhesion molecules are no longer expressed. The subsequent remodeling is probably less dependent on monocytes and mechanical factors. The phase of *maturation* begins between 14 days and 21 days after occlusion. It is charac-

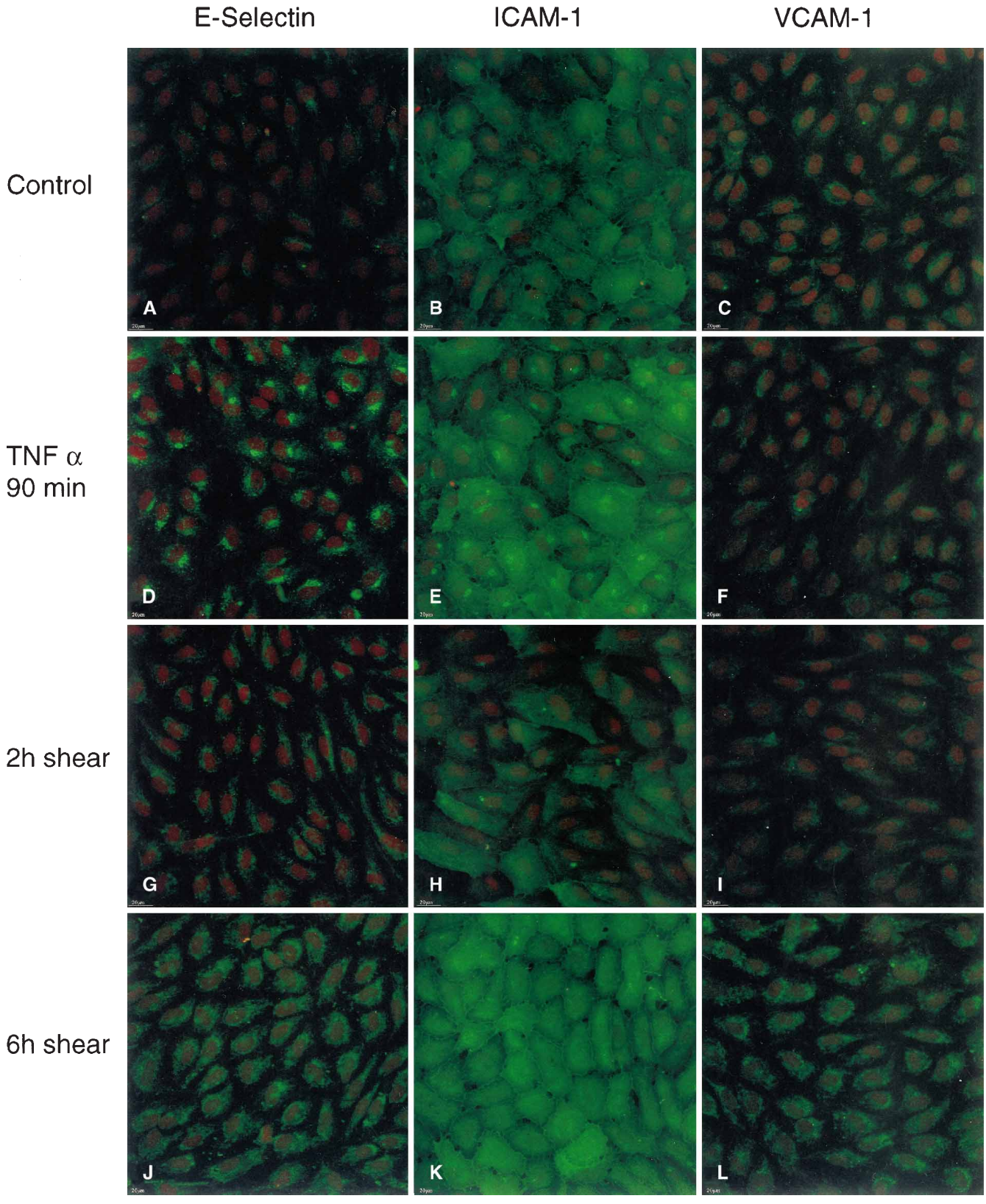


Fig. 10 Shear stress versus tumor necrosis factor (TNF)-α upregulation of endothelial adhesion molecules in vitro (HUVEC); nuclei are dark red stained with 7AAD; final magnification ×240. **A–C** Control; no E-selectin and VCAM-1 expression, low rate of a constitutive intercellular adhesion molecule (ICAM-1) expression. **B–F** Positive control: 90 min after 100 UE/ml TNF-α administration; perinuclear (corresponds rough endoplasmic reticulum and Golgi apparatus) staining for E-selectin and ICAM-1 suggests their new synthesis; too early for the vascular cell adhesion molecule (VCAM-1) expression [40]. **G–I** 2 h rotatory shear stress 12 dyn/cm^2; weak or no effect. **J–L** 6 h rotatory shear stress 12 dyn/cm^2; perinuclear staining suggests the new synthesis of all three adhesion molecules

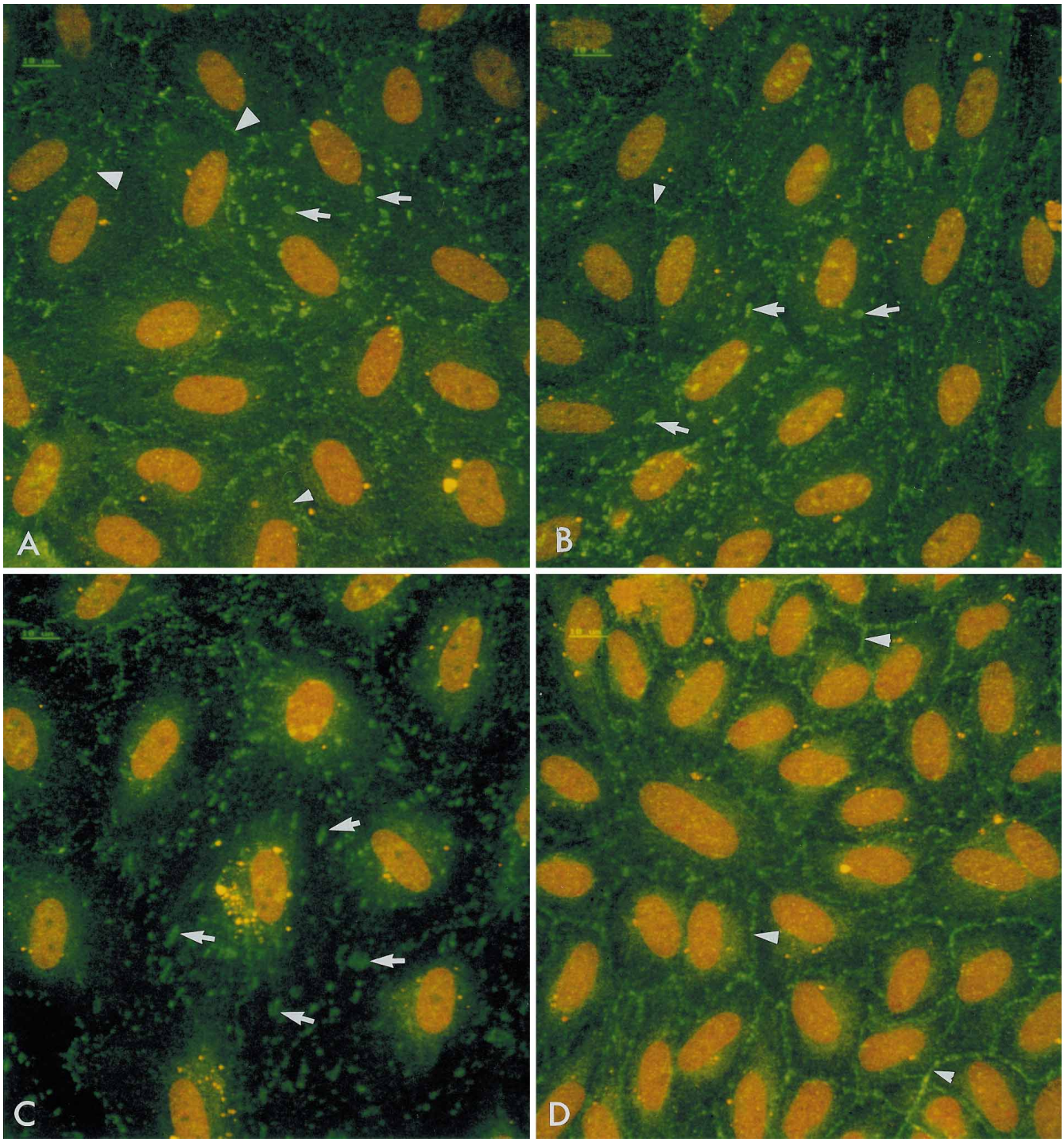


Fig. 11 Tyrosine phosphorylation of primary isolated endothelial cells (HUVEC) in control (A) and after 20 min (B), 30 min (C), and 14 h (D) rotatory shear stress of 12 dyn/cm^2. After 20–30 min shear stress, cell-to substrate focal adhesion contacts (*arrows*) became more strongly and cell-to-cell contacts (*arrowheads*) more weakly phosphorylated. In contrast, there were no phosphorylated adhesion contacts after 14 h shear stress (final magnification ×640)

terized by low levels of proliferation, migration, and proteolytic activity and restoration of most SMCs to the contractile phenotype.

Arterial growth and blood supply

During the remodeling, collateral vessels increase their lumen five- to eightfold and so achieve about one quarter (it corresponds about 1/16 of section area) of the original femoral artery. Since 5–20 collateral arteries are devel-

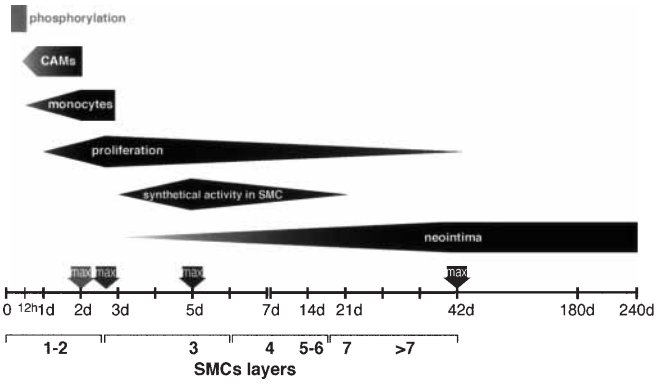


Fig. 12 Phases and events in the arteriogenesis

oped [27], their common section area could correspond to that of the femoral artery, and so we can believe in improved function. However, the blood supply through 20 small vessels should be lower than through the large one with the same section area, and cork-screw morphology would also diminish blood flow. The MCP-1 treatment did not change collateral diameters. This finding was expected because MCP-1 increases number rather than diameter of collateral vessels [26].

Comparison with other animal models of arteriogenesis

The canine model of coronary collateral remodeling was developed by our group years ago [36, 37]. Many of the histological findings from that model could be reproduced in the rabbit model, i.e., the initial thinning of pre-existing arterioles, the monocyte invasion, the intima formation, and the final disappearance of the majority of initially remodeling vessels in favor of a few large ones. However, no statements could be made with regard to the mechanisms behind arterial growth, because the molecular tools were not available at that time. Today, we can claim that the principles of arteriogenesis are probably not different between different organs, with the histological identity of coronary and femoral collaterals as partial evidence. The advantage of the rabbit hind-limb model is the possibility to study both arteriogenesis and angiogenesis, since both are spatially separated because capillary growth occurs in the ischemic calf muscles whereas arteriogenesis proceeds in the non-ischemic upper thigh muscles [5].

From shear stress to the expression of adhesion molecules

We have proposed that shear stress may be the initiating factor for arterial growth. We hypothesize that pre-existing arterioles connecting two adjacent vascular territories experience increased shear stress when one of the supplying arteries is occluded, since the difference in pressure will increase blood flow velocity and hence shear stress in the interconnecting arterioles. This then may lead to increased expression of adhesion molecules and of MCP-1, both culminating in monocyte invasion. To test this assumption, we subjected cultured endothelial cells to shear stress and found that indeed the expression of ICAM-1 and of VCAM-1 was increased. Furthermore, our data suggest that fluid shear stress elicits the phosphorylation of focal contacts on the "abluminal" side within 20–30 min after onset of shear stress elevation and synthesis and expression of adhesion molecules on the upper "luminal" side after 2–6 h. Our previous in vitro study revealed that endothelial cells upregulate E-selectin, ICAM-1, and VCAM-1 1–2 h after stimulation with interleukin-1β or TNF-α [40]. The newly synthesized proteins can be detected on the cell surface after 4 h [40]. This time course correlates well with our present in vitro and in vivo findings. Upregulation of adhesion molecule expression was only observed in response to high levels of shear stress (12 dynes/cm^2 but not 3 dynes/cm^2) in our in vitro study. Not only shear stress, but other mechanical stimuli, such as stretching, upregulate ICAM-1 mRNA in endothelial cells [14]. It was recently shown that both shear stress and mechanical stretching in vitro increase the activities of a number of kinases to modulate the phosphorylation of many signaling proteins at focal adhesion sites [15]. We hypothesize that the activation of the mechanoreceptors at the focal contacts of endothelial cells by shear stress can be an early trigger in the chain of events leading to collateral remodeling.

The endothelial cell can be viewed as a membrane stretched over a frame composed of microtubules, intermediate filaments, and actin fibers, which transverse the cells and appear to end in characteristic adhesion complexes. Even under non-stimulated conditions, the entire endothelial cytoskeleton is maintained under tension and, in response to an externally applied stimulus, intracellular tension is redistributed over the cytoskeletal network. This "tensegrity architecture" within cells permits forces to be directly transmitted from the cell surface, through the cytoskeleton, across physically interconnecting filaments to the nucleus (for review see [25]). Thus, extracellular forces are superimposed upon pre-existing forces within cells attached to the extracellular matrix at focal adhesion points and to each other at cell-to-cell contacts. Generally, signaling molecules are clustered around and inherent to these contact sites so that it is conceivable that the application of a stress, which is transmitted through the entire cell by the actin cytoskeleton, activates signal-transduction cascades without the need of a specific shear stress or stretch receptor. Recently, molecular connections between integrins, cytoskeletal filaments, and nuclear scaffolds have been proposed to provide a pathway for signal transfer, thus raising the possibility that mechanical stimuli may be passed on to the nucleus in the absence of/or simultaneously with mechanochemical signaling processes [30]. We therefore assume that arteriolar endothelial cells (1) are shear stress sensors, (2) upregulate adhesion molecules, and (3) recruit monocytes.

From endothelial activation to monocyte recruitment and vascular proliferation

Cell adhesion molecules were found on the endothelial cells in a subsection of collateral arterioles as soon as 8–12 h after femoral occlusion. Perivascular accumulation of macrophages started after 12 h; mitotic activity of endothelial cells and SMCs after 24 h. In our previous study, we identified monocytes/macrophages as the source of the growth factors bFGF and TNFα, which can initiate the proliferation of endothelial cells, SMCs, and fibroblasts in the collateral vessel wall [1]. From the present in vivo data, we can add that the maximal proliferative activity coincided with a peak in the accumulation of monocytes between days 2 and 3 after occlusion. In vitro data suggest that a delay of 8–12 h occurs between the stimulation of endothelial cells with bFGF and the start of proliferation. These observations correlate well with the data obtained in vivo, where we detected monocytes/macrophages around collaterals 12 h prior to the beginning of proliferation. The reason for such a delay may be the time required for the transition of cells from the G phase to the G1 phase of the cell cycle. Expression of the Ki-67 antigen is a standard marker of cell proliferation [12, 23, 39, 41]. In separate studies with endothelial cells in culture (results not shown), we established that about 8 h are needed following mitotic stimulation before Ki67-positive cells became detectable.

Endothelial cells and SMCs began to proliferate (assessed by Ki-67) 24 h after femoral occlusion. The number of labeled cells is maximal at 2–3 days, was slightly reduced at 7 days, but remained detectable until day 42 (Fig. 9 and Fig. 12). This time course is in agreement with the recently described mouse model of peripheral arteriogenesis [18]. Six to eight months after the occlusion, no signs of proliferation were observed, which agreed with the diameter measurements.

Effect of MCP-1

The endothelium-derived chemokine MCP-1 specifically recruits monocytes from the blood stream [44]. An abundance of blood-derived monocytes as late as 7 days after femoral occlusion was found only in MCP-1-treated rabbits, which correlated with accelerated collateral growth (described in [27]). We suppose that MCP-1 prolongs the survival of blood-derived monocytes and, therefore, enhances the secretion of growth factors. In the present study, we observed that treatment with MCP-1 increased the number of blood-derived macrophages around collaterals and prolonged the period of detection (up to 7 days). In the absence of MCP-1 treatment, blood-derived monocytes were observed only up to day 3. Comparing this with the ultrastructural findings, we propose that after this period the macrophages either die or are transformed. Other substances promoting monocyte survival, such as the granulocyte–macrophage colony-stimulating factor (GM-CSF), also enhance the growth of collateral vessels [9].

Neo-intima formation

A neo-intima appeared as early as day 3 after occlusion as a thin layer of SMCs between the lamina elastica interna and the endothelium. It develops in the midzone section of the collateral artery. The SMCs of the neo-intima were not circularly, but helically directed; they were contracted and enveloped in elastic material. The neo-intima develops first at the sites of SMC migration and correlates with the degree of lamina elastica interna disruption. We presume that the lamina elastica interna must be dissolved before SMCs can migrate into the intima and indicate that this process is spatially restricted and may involve matrix metalloproteinases [11]. Electron microscopic and immunohistochemical data suggest that all cells in the neo-intima were SMCs, as described originally in the intimal injury model [43]. Neo-intima development as a result of the SMC proliferation has also been described in rabbits following carotid artery catheter angioplasty [3], stent implantation into the iliac artery [4], and in atherosclerosis [13]. Inhibition of nitric-oxide synthases accelerates the formation of the neo-intima [33], while inhibition of the renin–angiotensin system slows it down [17]. In contrast to the three latter models, in collateral remodeling, the diameter as well as conductance of arteries increases despite a prominent neo-intima.

Conclusion

Following occlusion of the femoral artery, endothelial cells in collateral vessels become activated as evidenced by phosphorylated focal contacts, upregulation of adhesion molecules, and ultrastructural changes. Monocytes accumulate, which is followed by arterial remodeling in which phases of initiation, proliferation, synthesis, and maturation could be distinguished by morphological and molecular markers. In vitro data suggest that the mechanical stimulation of endothelial cells by shear stress can be a primary trigger in the chain of events leading to arteriogenesis.

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