Arteriogenesis Proceeds via ICAM-1/Mac-1–Mediated Mechanisms

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Abstract—Monocyte adhesion to shear stress–activated endothelium stands as an important initial step during arteriogenesis (collateral artery growth). Using multiple approaches, we tested the hypothesis that monocyte adhesion via intercellular adhesion molecule-1 (ICAM-1) and selectin interactions is essential for adaptive arteriogenesis. Forty-eight New Zealand White rabbits received either solvent, monocyte chemoattractant protein-1 (MCP-1) alone, MCP-1 plus ICAM-mab, or MCP-1 plus an IgG2a isotype control via osmotic minipumps. After 7 days, collateral conductance was evaluated: solvent 4.01 (mL/min per 100 mm Hg), MCP-1 plus ICAM-mab 8.04 (versus solvent \( P=NS \)), and MCP-1 alone 33.11 (versus solvent \( P<0.05 \)). Furthermore, the right femoral arteries of ICAM-1–\(^{-/-}\), Mac-1–\(^{-/-}\) and mice having defective selectin interactions (FT4/7\(^{-/-}\)) as well as their corresponding controls were ligated. One week later, perfusion ratios were determined by the use of fluorescent microspheres. FT4/7\(^{-/-}\) mice did not show any significant difference in perfusion restoration whereas ICAM-1–\(^{-/-}\) and Mac-1–\(^{-/-}\) mice had a significant reduction in arteriogenesis as compared with matching controls (FT4/7-WT 37±9%, FT4/7\(^{-/-}\) 32±3%, \( P=0.31 \); C57BL/6J 59±9%, ICAM-1–\(^{-/-}\) 36±8%, \( P<0.05 \); Mac-1–\(^{-/-}\) 42±3%, \( P<0.05 \)). ICAM-1/Mac-1–mediated monocyte adhesion to the endothelium of collateral arteries is an essential step for arteriogenesis, whereas this process can proceed via selectin interaction independent mechanisms. Furthermore, in vivo treatment with monoclonal antibodies against ICAM-1 totally abolishes the stimulatory effect of MCP-1 on collateral artery growth, suggesting that the mechanism of the MCP-1–induced arteriogenesis proceeds via the localization of monocytes rather than the action of the MCP-1 molecule itself. (Circ Res. 2004;94:1179-1185.)

Key Words: collateral circulation ■ cell adhesion molecules ■ microspheres ■ blood flow ■ intercellular adhesion molecule-1

Networks of arterioles that interconnect perfusion territories exist in many regions of the body (eg, heart, brain, extremities).\(^{1-3}\) In the event of arterial occlusion or a slowly progressing stenosis, a steep pressure gradient develops along the shortest path within the interconnecting network that increases blood flow velocity and hence fluid shear stress in these vessels that now assume their new function as collateral arteries. These vessels then grow to provide enhanced perfusion to the jeopardized ischemic regions. This adaptive process is termed arteriogenesis\(^{4,5}\) and refers to active proliferation and remodeling rather than to passive dilatation.

The effect of the sustained increase in shear force is the activation of the endothelium with subsequent upregulation of cell adhesion molecules, in particular intercellular adhesion molecule-1 (ICAM-1),\(^{6-8}\) increased endothelial production of cytokines (MCP-1, GM-CSF, TNF-\(\alpha\)),\(^{9}\) attraction of circulating monocytes to the activated endothelium, and finally their adhesion and invasion with subsequent matura-
tion into macrophages.\(^{10}\) These in turn create an inflammatory environment and produce increased amounts of growth factors.\(^{11,12}\) The selectins also stand as known early mediators of monocyte adhesion,\(^{13}\) although their role in arteriogenesis has not been defined.

This early localization of monocytes is soon followed by the first wave of mitosis of the endothelial- and vascular smooth muscle cells (proliferating phase). Besides mitosis, the perivascular inflammation creates the space for the greatly expanding collateral vessels that can increase their diameter markedly depending on animal size (20-fold in the canine heart, 7-fold in the rabbit hind limb). The old structure is essentially dismantled and replaced by new medial smooth muscle cells (remodeling phase), resulting in a functional artery originating from a preexisting arteriole.\(^{14,15}\)
In previous studies, we have shown that chronic intraarterial infusion of the monocyte chemoattractant protein-1 (MCP-1) and granulocyte-macrophage colony-stimulating-factor (GM-CSF) increased the development of arterial collateral blood vessels after femoral artery occlusion. In another study, a single dose of lipopolysaccharide (LPS) was injected intravenously into New Zealand White rabbits 3 days after ligation of the femoral artery. LPS is a potent stimulator of tumor necrosis factor-α (TNF-α), which is a key mediator of arteriogenesis. The LPS injection enhanced the number of monocyte-derived macrophages that accumulated around growing collateral arteries. Peripheral and collateral conductances were markedly increased. Histological studies showed an upregulation of ICAM-1 on the endothelium of the newly recruited collateral arteries as compared with normal arteries. However, it remained unclear whether this ICAM-1 upregulation was a coincidental or pivotal process in this setting. In addition to the upregulation of ICAM-1 on the endothelial surface of growing collateral arteries, recent data suggested that the mechanism of transendothelial migration is mediated by ICAM-1/Mac-1 interaction, as proarteriogenic factors significantly enhanced the expression and presentation of this receptor.

Thus, in an effort to specifically define the molecular mediators of collateral artery development, we hypothesized that arteriogenesis proceeds by way of ICAM-1/Mac-1 interaction and selectin dependent mechanisms. In this study, we demonstrate that the stimulatory effect of monocyte chemotraction on collateral artery growth (arteriogenesis) is reduced in case of ICAM-1 or Mac-1 deficiency or by monoclonal antibodies against ICAM-1, and that this process can proceed independent of selectin interactions.

Materials and Methods

The present study was performed after securing appropriate institutional approvals. It conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Specificity Testing of the Monoclonal Antibody Against ICAM-1 via Western Blot Analysis

A mouse IgG2a-antibody directed against domain 2 of human ICAM-1 was used to disrupt ICAM-1-mediated leukocyte adhesion. It inhibits the interaction of both CD11a/CD18 and CD11b/CD18 with ICAM-1. To verify its specificity, six rabbit aortas (Charles River, Sulzfeld, Germany) were isolated, homogenized (Sonifier 20W, 5 minutes), and lysed for 30 minutes at a temperature of 4°C (150 mmol NaCl; 50 mmol Tris-HCl; 0.1% SDS; 1% Triton X-100; 100 μg/mL PMSF). Lysates were cleared by centrifugation for 30 minutes at 10,000 g, then proteins (10 μg/slot) from the supernatant were dissolved in Laemmli’s buffer, separated via SDS-PAGE (10% polyacrylamide gel), and then blotted onto a nitrocellulose membrane. To block nonspecific binding, the membrane was incubated with 10% skim milk powder in PBS-Tween (0.01%) for 1 hour. After washing, the monoclonal antibody against ICAM-1 was added at a concentration of 10 μg/mL for 2 hours at room temperature in PBS-Tween. The membrane was then immunoprobed with HRP-conjugated goat-anti-mouse-IgG antibodies (1 μg/mL). An enhanced ECL-kit was applied to detect the target protein ICAM-1 and human leukocytes from healthy volunteers served as control.

Activity Testing of the Monoclonal Antibody Against ICAM-1 via Monocyte Adhesion Assay

In order to verify binding of this mouse-anti-human monoclonal antibody to the antigen, we isolated rabbit peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation using Ficoll-Hypaque (Pharmacia & Upjohn). Monocytes were further isolated from PBMC by counterflow- centrifugation with a Beckman JE-6B elutriator.

Monocyte-enriched fractions contained 90% monocytes as determined by FACS analysis via CD14 antigen expression. Monocytes were cultured for 16 hours in serum-free macrophage medium (Life Technologies) on Petri Perm culture plates (Heraeus). Stimulation of monocytes was performed by incubation with MCP-1 (100 ng/mL, 4 hours, 37°C, Peprotech, UK). For adhesion assays, tissue culture plates were coated with either soluble ICAM or VCAM-1 (4 μg/mL, 90 minutes, 37°C, RND systems). Plates coated with PBS/BSA (0.1%) served as control. After washing with PBS (pH 7.4) plates were blocked with PBS/BSA (1%) for 30 minutes.

Rabbit Model

Forty-eight New Zealand White rabbits (NZWR) were randomly assigned to either receive monocyte chemoattractant protein-1 (MCP-1, 3 μg, Peprotech), MCP-1 plus supplementary mouse IgG2a-monoclonal antibody against ICAM-1 intravenously (3 mg/kg per day), MCP-1 plus supplementary nonspecific IgG2a isotype control antibody or PBS intra-arterially via osmotic minipumps as previously described. The body weights and body temperature in rabbits treated with MCP-1 and the combination of MCP-1 with mab-ICAM-1 did not significantly differ from those of control rabbits.

Postmortem Angiography

After maximal vasodilation of hind limbs by infusion of adenosine (1 mg/kg body weight) into the abdominal aorta, legs were perfused with PBS in a warmed water bath of 37°C for 1 minute at a pressure of 80 mm Hg, followed by perfusion for 8 to 10 minutes at 80 mm Hg with contrast medium based on bismuth and gelatin according to a formula developed by Fulton.1 Angiograms were taken at two different angles and the resulting stereoscopic images were used for 3-dimensional analysis of collateral growth.

Quantification of Collateral Arteries

Collateral arteries were classified according to Longland26 into stem, midzone, and reentry using stereoscopic viewing of angiograms at 3-fold magnification. Collateral arteries then were divided into two groups: group one consisted of vessels whose stem branched from the artery circumflexa femoris. Group two of the arteries originated from the artery profunda femoris. The length of the midzone in each group was almost the same, so their measurement did not give any further information. Collateral arteries from the first group usually reentered the arteria genus descendens, the second group into the arteria caudalis femoris. Only about 10% of the collateral arteries originate from other vessels, eg, from the a. iliaca externa or from the a. iliaca interna (Figure 3D). Collateral vessels were marked after counting to insure that no vessel was counted twice.

Histemodynamic Measurements

Collateral conductance was quantified as previously described. The carotid artery, the abdominal aorta, and both saphenous arteries were cannulated and a pump-driven shunt between carotid artery and aorta was installed. Hind limbs were perfused under maximal vasodilation (adenosine 1 mg/kg per minute) at different pressure levels, each combined with the injection of a differently labeled fluorescent microsphere. Peripheral and systemic pressures as well as systemic blood flow were recorded (PowerLab) for further analysis. Tissue samples were harvested and digested for FACS analysis for quantification of the microspheres. Collateral conductance was calculated from the slope of the pressure-flow relations.
Mouse Model
The right femoral arteries of nine ICAM-1−/− (B6.129S7-Icam1tm1Bay) (N12 backcross onto C57BL/6J, Jackson Laboratory, Bar Harbor, Maine), seven Mac-1−/− (B6.129S7-Ifgib2tm1Bay) (N9 backcross onto C57BL/6J, Jackson Laboratory, Bar Harbor, Maine), six α(1,3)fucosyltransferase (FT) FT4 and FT7 deficient mice (FT4/7−/−; Jack P and E sialyL Lewis x counter-receptor oligosaccharide and possess deficient L-selectin ligand activity27 (source John B. Lowe, University of Michigan), as well as their corresponding controls (C57BL/6J, n = 7, Jackson Laboratory, Bar Harbor, Maine, and FT4/7-WT, n = 6, derived from CD57BL/6JxDBA/2J mice, detailed description in Homeister et al27) were ligated directly distal to the inguinal ligament. After 1 week, perfusion ratios of occluded versus nonoccluded hind limbs were evaluated using fluorescent microspheres as previously described.19 The abdominal aorta was cannulated and both hind limbs were perfused under maximal vasodilation combined with the injection of differently labeled fluorescent microspheres at different perfusion pressure levels. Microspheres were quantified using FACS-analysis and flow ratios were calculated. All results are expressed as perfusion of occluded versus nonoccluded hind limb. Furthermore, groups of ICAM−/− and C57BL/6 mice were analyzed 3 days (n = 7 each) and 14 days (n = 6 each) after femoral artery ligation to study the time course of arteriogenesis in these mice.

Immunohistochemistry
An additional six C57BL/6 and six ICAM-1−/− mice was ligated as described above. Seven days later, tissue samples from the thigh were taken and snap frozen for subsequent immunohistochemistry. Immunohistological stainings for monocytes were performed as previously described20 using a monoclonal antibody against MOMA-2 (BMA Biomedicals). Growing collateral arteries were identified by Ki-67 staining (Figure 6A). A total of 12 sections per animal was stained and macrophage content was quantified by a single blinded observer. The amount of macrophages is expressed as the number of MOMA-2-positive cells per mm².

Statistical Analysis
Data are described as mean±SD. Differences were assessed using post hoc ANOVA and Bonferroni correction. Values of P ≤ 0.05 were accepted for statistical significance.

Results
All animals were clinically healthy and showed no obvious defects. None of the animals were lost during or after femoral artery ligation. We also did not observe any gangrene or gross impairment of hind limb function after femoral artery occlusion. The remaining volume in the minipumps was evaluated in all groups to ensure complete delivery and was 0.31±0.08 mL.

Verification of the Antiadhesion Effect of the Monoclonal Mouse IgG-Antibody Against ICAM-1
The anti-ICAM-1 mab bound specifically to rabbit ICAM-1 as shown by Western Blot analysis (Figure 1). The efficacy of the anti-ICAM-1 mab was proven via a monocye adhesion assay. Isolated human as well as rabbit monocytes exposed to MCP-1 showed a significant increase in adhesiveness to immobilized ICAM-1 as compared with PBS-treated monocytes. The addition of the ICAM-1-mab significantly reduced the amount of adhering cells to the level comparable to PBS, whereas it did not affect cell adhesion to VCAM-1 (Figure 2). Thus, the immobilized human ICAM-1 is recognized by the rabbit monocyte ligand and binding of the mouse anti-human ICAM-1 antibody to its coated ligand prevents adhesion of rabbit as well as human monocytes.

Rabbit aorta
Human leukocytes
Human ICAM-1

Figure 1. Verification of the antibody’s specificity to rabbit ICAM-1 by Western blot analysis. Thirty micrograms of protein from lysed rabbit aorta and 500 ng sICAM were separated by SDS-PAGE and blotted. Specific immunostaining was performed using mab anti-ICAM-1. Human leukocytes served as an additional control.

Radiographic Findings
Postmortem angiograms were performed to study the effect of blocking leukocyte adhesion under conditions of MCP-1 stimulation on collateral artery growth. Corkscrew-like collaterals are located mainly in the adductor longus-, adductor magnus-, and vastus intermedius muscles and can be divided in two groups: group one consisted of vessels whose stem branched from the arteria circumflexa femoris lateralis. Group two of the arteries originated from the arteria profunda femoris. Angiograms taken from hind limbs of animals treated with MCP-1 alone (31.16±2.34 number of visible collateral arteries) (Figure 3B) showed a statistically significant increase in the diameter and number of visible collateral vessels. This increase declined to values comparable after PBS treatment (15.83±1.8) (Figure 3A), due to the additional application of monoclonal antibodies against ICAM-1 (18.30±1.76) (Figure 3C). To further examine the functional aspects of the recruited collateral network, hemodynamic measurements under maximal vasodilation were performed using fluorescent microspheres for the calculation of collateral conductance.

Collateral Conductance
After MCP-1 treatment, the conductance value showed a significant increase (33.11±1.98 mL/min per 100 mm Hg) as compared with the solvent-treated group (4.01±0.58). This increase declined to normal values under simultaneous application of monoclonal antibodies against ICAM-1 (8.04±2.52) (Figure 4). The addition of a nonspecific isotype control antibody to MCP-1 in contrast did not significantly alter the influence of MCP-1 on collateral conductance (31.81±2.52). This indicates that the stimulatory effect of MCP-1 on arteriogenesis is directly reduced by inhibiting...
leukocyte migration through infusion of the ICAM-1 antibody.

**Perfusion Restoration in Transgenic Mice**

Transgenic mice were used to quantify the “natural” arteriogenic response without MCP-1 stimulation in the absence of single proteins. FT4/7−/− mice did not show any significant difference in perfusion restoration 1 week after femoral artery ligation as compared with wild-type controls (FT4/7−/−: 32±3%; FT4/7-WT: 37±9%; P=0.31). In contrast, ICAM-1 deficiency led to a significant reduction in the arteriogenic response to the acute occlusion of the femoral artery as

Figure 2. A and B, Verification of the adhesion inhibition by monoclonal mouse IgG antibody against ICAM-1. Addition of the monoclonal antibody significantly reduces the number of adhering monocytes, whereas a nonspecific control antibody does not significantly affect monocyte adhesion to ICAM-1 (Figure 2A). Treatment with the anti–ICAM-1 antibody did not significantly affect monocyte adhesion to VCAM-1 (Figure 2B), confirming its specificity for ICAM-1.

Figure 3. A through C, Postmortem angiograms after 7 days of continuous infusion of PBS (A): only very few collateral arteries can be identified; MCP-1 (B): significant increase in the number as well as the density of collateral arteries, collateral arteries with typical corkscrew formation can be identified; MCP-1 plus monoclonal antibody against ICAM-1 (C): the anatomic findings are comparable to the PBS treatment; control hind limb without femoral artery ligation (D): the arrows indicate the 2 main stem branches from which collateral arteries originate in this model. Number of visible collateral arteries has been quantified under stereoscopic view identifying each collateral vessel spanning the occlusion site. Source vessels are the circumflex femoral artery for vessels located in the *Musculus* quadriceps femoris and the profound femoral artery for vessels located in the adductor and abductor muscles.
expressed by reduced perfusion ratios (ICAM-1−/−: 36 ± 8%; C57BL/6/J: 59 ± 9%; P < 0.001). Mac-1−/− mice showed a comparable reduction in perfusion restoration at day 7 after femoral artery occlusion (Mac-1−/−: 42 ± 3%; P < 0.05 versus control). Perfusion ratios at day 3 after femoral artery occlusion already showed slight but not significant differences between ICAM-1−/− and backgrounds (ICAM-1−/−: 12 ± 1%; C57BL/6/J: 16 ± 4; P = 0.06), which were still present at day 14 (ICAM-1−/−: 57 ± 9%; C57BL/6/J: 74 ± 10; P < 0.05) (Figure 5). Thus, the firm adhesion of leukocytes to ICAM-1 mediated by ICAM-1/Mac-1 interaction is essential for the natural time-course of arteriogenesis.

Monocyte Quantification in Mice
Because monocytes/macrophages are supposed to be one of the most important cellular factors during arteriogenesis, monocyte/macrophage content was determined in ICAM-1−/− mice. The amount of transmigrated macrophages that stained positive for MOMA-2 in the surrounding tissue of the growing collateral arteries of mice 7 days after femoral artery ligation revealed a significantly reduced number of monocytes/mm² in ICAM-1−/− mice as compared with the genetic backgrounds (ICAM-1−/−: 12.80 ± 6.46; C57BL/6/J: 19.75 ± 5.26 positive cells/mm²; P < 0.05) (Figure 6). These data confirmed our hypothesis that monocyte migration is significantly reduced in mice lacking functional ICAM-1.

Discussion
The active recruitment of preexisting collateral arteries is the most efficient adaptive mechanism for the survival of ischemic limbs or internal organs such as the heart and brain because of its ability to conduct, after adaptive growth, relatively large blood volumes per unit of time.28 Previous studies showed that local infusion of MCP-1 into the collateral circulation after femoral artery occlusion increases collateral conductance in the rabbit.16,25 Histological and angiographic results suggested that this increase in conductance was mainly due to an enhanced chemotactic recruitment of monocytes that, in turn, deliver growth factors, which lead to the proliferation of endothelial and smooth muscle cells.

In the present study, we report that this stimulatory effect of MCP-1 on arteriogenesis18 can be inhibited with monoclonal antibodies against endothelial ICAM-1 in vivo,22,29 indicating that monocyte binding to ICAM-1, adhesion to the endothelium, and subsequent invasion is an obligatory and important step for the arteriogenic action of MCP-1 and hence monocytes. On the other hand, our data indicate that arteriogenesis can proceed independent of selectin-mediated interactions, as demonstrated by the results from the FT4/7−/− animals. For quantification of hemodynamic changes, we used an in vivo model under conditions of maximal vasodilatation to reveal the result of anatomic remodeling undisturbed by autoregulatory vasomotion.

ICAM-1 plays a crucial role during monocyte adhesion. This surface glycoprotein (molecular weight 75 to 114 kDa, depending on different glycosylation forms30) belongs to the immunoglobulin superfamily, is expressed on the endothelium and promotes the adhesion of monocytes, neutrophils, and lymphocytes.31,32 ICAM-1 interacts with monocytes principally by binding to the surface membrane β2 integrin molecule lymphocyte function–associated antigen-1 (LFA-1)33 and also by binding to the β2-integrin CD11b/CD18 receptor (Mac-1).34–36 Besides shear stress,7,37,38 several inflammatory mediators (eg, MCP-1, TNF-α, LPS) enhance both endothelial ICAM-1 expression in vitro and at sites of inflammation in vivo and Mac-1 receptor expression on monocytes in vitro.39 Furthermore, immunohistochemistry of collateral arteries shows a significant expression of ICAM-1 after experimentally induced femoral artery ligation, with confirmation via marked upregulation of endothelial ICAM-1 mRNA after femoral artery occlusion.15 Previous experiments showed that monoclonal antibodies against ICAM-1 inhibit leukocyte adhesion and transmigration in vivo and in vitro.31,40

Several studies indicate that the loss of one particular adhesion molecules is usually of limited biological significance and two or more different adhesion molecules are needed for optimal leukocyte adhesion.32,41 Initial monocyte/endothelial cell adhesion (specifically “rolling”) is mediated by P and E selectin through a sialyl Lewis x counter-receptor.42,43 Biosynthesis of this oligosaccharide is controlled by α(1,3)fucosyltranscrases FT4 and FT7.27

Figure 4. Collateral conductance in the rabbit hind limb 7 days after femoral artery ligation as assessed by combined pressure and flow measurements with fluorescent microspheres. ns indicates not significant.

Figure 5. Perfusion restoration in transgenic mice. All results are expressed as a percentage of perfusion per gram of tissue in the lower limb of the occluded hind limb vs perfusion of the corresponding tissue in the nonoccluded hind limb. Nonoccluded hind limb thus serves as an internal standard of normal perfusion.
On femoral artery ligation, FT4/7/−/− mice did not show significant differences in flow restoration as compared with controls. These data are consistent with other studies showing no significant decrease in mononuclear leukocyte adhesion to endothelium in a functionally similar strain,44 because further adhesion molecules are needed (eg, ICAM-1). In this context, surprisingly, the functional defect of only ICAM-1 leads to a significant reduction of the arteriogenic response to the ligation of the femoral artery. Thus, ICAM-1 is not only important for stimulation of arteriogenesis induced by the infusion of monocyte chemoattractants, but it also appears essential for monocyte adhesion and transmigration during the time course of collateral artery growth. These data were confirmed in mice lacking the counterpart of the endothelial ICAM-1 on the leukocyte, the Mac-1 receptor, which showed a comparable reduction in perfusion restoration. It should however be noted that the results from the ICAM-1/−/− cannot directly be compared with the perfusion ratios from the FT4/7/−/− mice, as both strains were derived from different background strains. Thus, perfusion data of the single strains are only conclusive by comparing the KO animals with the corresponding genetic background.

Conclusions

This study provides direct evidence that ICAM-1–mediated monocyte adhesion via ICAM-1/Mac-1 interaction to the endothelium of collateral arteries is an essential step for arteriogenesis, whereas this process can proceed via selectin interaction independent mechanisms. Furthermore, in vivo treatment with monoclonal antibodies against ICAM-1 totally abolishes the stimulatory effect of MCP-1 on collateral artery growth, indicating that the mechanism of the MCP-1 induced effect refers to the invasion of monocytes rather than the action of the MCP-1 molecule itself. Monocyte invasion, the sequel of ICAM-1 upregulation on the endothelial side and the first detectable morphological event in early collateral artery formation, stands as a pivotal early event in arteriogenesis. Further studies are on the way to evaluate the role of ICAM-1 in the recruitment of subpopulations of circulating mononuclear (pluripotent) stem cells.

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