Crucial Role of the CCL2/CCR2 Axis in Neointimal Hyperplasia After Arterial Injury in Hyperlipidemic Mice Involves Early Monocyte Recruitment and CCL2 Presentation on Platelets

Andreas Schober, Alma Zernecke, Elisa A. Liehn, Philipp von Hundelshausen, Sandra Knarren, William A. Kuziel, Christian Weber

Abstract—Monocyte chemoattractant protein-1 (also known as CC chemokine ligand 2 [CCL2]) and its receptor CC chemokine receptor 2 (CCR2) play a central role in the inflammatory response and neointimal formation after vascular injury. In the context of hyperlipidemia, this appears to involve neointimal monocyte infiltration. Hence, we investigated the function of the CCL2/CCR2 axis in early monocyte recruitment to injured arteries. Wire-induced injury of the carotid artery in apoE−/− mice caused a rapid increase of JE/CCL2 protein in the vessel wall peaking at 24 hours after injury, whereas serum JE/CCL2 was increased solely at 6 hours and blood cell-associated levels were unaltered, as demonstrated by enzyme-linked immunosorbent assay. Immunohistochemistry revealed intense staining for JE/CCL2 in smooth muscle cells (SMCs) and in association with platelets adherent to the denuded vessel wall 24 hours after injury. In vitro, exogenous or SMC-derived JE/CCL2 binds to the platelet surface and triggers monocyte arrest on adherent platelets but not on SMCs in flow assays. Accordingly, monocyte arrest in ex vivo perfused apoE−/− carotid arteries isolated 24 hours after injury was profoundly inhibited by pretreatment with a JE/CCL2 antibody. In CCR2−/−/apoE−/− mice, neointimal plaque area was reduced by 47% compared with CCR2+/−/apoE−/− mice. Moreover, CCR2 deletion markedly decreased neointimal macrophage content while expanding SMC content. Vascular JE/CCL2 expressed by SMCs and immobilized by adherent platelets after endothelial denudation is crucial for mediating early monocyte recruitment to injured arteries in hyperlipidemic mice. This mechanism may explain reduced neointimal macrophage infiltration and lesion formation in CCR2-deficient apoE−/− mice. (Circ Res. 2004;95:1125-1133.)

Key Words: inflammation ■ restenosis ■ platelets ■ vascular biology ■ monocyte chemoattractant protein-1

Acutely vascular injury initiates an inflammatory response attributable to recruitment of leukocytes on adherent platelets via P-selectin.1,2 Monocytes enter the emerging neointima and accumulate in the injury-induced lesion,2,3 providing a key stimulus for vascular repair, which may become excessive to cause relevant arterial stenosis. Hypercholesterolemia, a potent trigger of vessel wall inflammation in spontaneous atherosclerosis, enhances monocyte recruitment after periadventitial arterial injury and thus mediates the exacerbation of neointimal growth.5–8 In the context of hypercholesterolemia, locally expressed CCL2 can recruit monocytes into the vessel wall via CCR2.9,10 Because hypercholesterolemia induces CCL2 in smooth muscle cells (SMCs)11 and upregulates CCR2 on monocytes,12 this pathway is a prime suspect responsible for over-recruitment of leukocytes after acute vascular injury. Inhibition of CCL2, which is rapidly but transiently upregulated after arterial injury,13,14 reduced neointimal area and macrophage infiltration in hypercholesterolemic rabbits after angioplasty.15 Although most studies using normocholesterolemic animals13,14,16,17 report that inhibition of the CCL2/CCR2 axis diminished lesion size, monocyte accumulation was reduced only after periarterial cuff placement.16 Rather, genetic deletion of CCR2 in mice17 or a blocking CCL2 antibody in rats13 decreased neointimal SMC content. The role of CCL2/CCR2 in vascular repair may thus differ between normocholesterolemia and hypercholesterolemia.
Here, we investigated the effect of CCR2 deficiency on neointima formation and monocyte recruitment after wire-induced carotid artery injury in apoE−/− mice. Moreover, we studied the pattern and localization of JE/CCL2 expression early after injury, revealing its presentation by adherent platelets and its functional contribution to monocyte arrest.

Materials and Methods

Atherogenic Murine Model of Restenosis
CCR2−/−/apoE−/− mice (87.5% CS7Bl/6; 12.5% 129/Ola) were generated as described.6 CCR2−/−/apoE−/− mice from the same parental genotype served as control. Female 8-week-old mice were fed an atherogenic diet (21% fat, 0.15% cholesterol, 19.5% casein, wt/wt; Altromin, Lage, Germany) for 1 week before and 4 weeks after carotid injury. After anesthesia by intraperitoneal injection of ketamine hydrochloride/xylazine, a 0.36-mm flexible guidewire was advanced via transverse arteriotomy of the external carotid artery, and endothelial denudation was achieved by 3 passes along the left common carotid artery (CCA).18 Carotid arteries (n=5 each group) were excised 28 days after wire injury after in situ perfusion–fixation with 4% paraformaldehyde and paraffin-embedded. Blood and carotid arteries were collected before (0 hours) or after wire injury (6 hours, 24 hours, 48 hours; n >6 per group). Arteries were shock-frozen in liquid nitrogen or fixed with 4% paraformaldehyde. Animal experiments were approved by local authorities (Bezirksregierung Köln) and complied with the European Convention of Animal Care and German animal protection law. Serum cholesterol and triglyceride levels were determined by standard enzymatic assays (Roche).

Ex Vivo Perfusion of Murine Carotid Arteries After Wire Injury
After 1 week of atherogenic diet, female 8-week-old apoE−/− mice (M&B, Ry, Denmark) were subjected to wire injury. Carotid arteries were isolated 24 hours after denudation for ex vivo perfusion as described.19 In brief, CCA and the internal branch were ligated and a catheter was inserted through an incision of the CCA cranial to the suture. The artery was perfused with MOPS-buffered physiological salt solution (0.5% human serum albumin) using a syringe pump (GENIE; Kent Scientific Corporation, Litchfield). Outflow was enabled by punctures in the external and internal branches. The vessel was cut distal to sutures and transferred onto a microscope stage (saline immersion objective/20; Olympus BX51) superfused at 37°C with bicarbonate-buffered saline. Monocytic MonoMac6 (MM6) cells (250 000/mL) labeled with calcein-AM (Molecular Probes) or peripheral blood leukocytes (3×106/mL) from fms-enhanced green fluorescent protein (EGFP) mice expressing EGFP selectively in mononuclear phagocytes20 were perfused at 5 L/min, and adhesive interactions with the injured vessel wall (arrest, rolling) were recorded using stroboscopic epifluorescence illumination (Drelloscope 250; Drello, Mönchengladbach, Germany). Some arteries were preperfused with blocking JE/CCL2 antibody (Ab) 2H5 (Pharmingen; 50 µg/mL) for 20 minutes or an irrelevant control.
antibody (n=3 each group). Adhesion of monocytes in predefined areas of injured CCAs and unjured areas of internal carotid arteries was analyzed (see supplemental Figure I available in the online data supplement at).

Quantitative Histomorphometry, Immunohistochemistry, and Immunofluorescence
Serial tissue sections (5 μm) were obtained from the left CCA, starting at the bifurcation. Ten sections per mouse (50 μm apart) were stained using Movats-modified pentachrome.18 Areas within lumen and internal and external elastic laminae were measured by planimetry of digitized images using Diskus Software (Hilgers, Königswinter, Germany) to determine neointimal and medial areas. Sections were reacted with Abs to Mac-2 (M3/38, Cedarlane), α-smooth muscle cell actin (1A4; Dako), collagen type I (Cedarlane), or isotype controls. JE/CCL2 was detected by a goat polyclonal Ab (Santa Cruz) at 4°C overnight. In adjacent sections, staining for P-selectin (rabbit P-selectin Ab; Pharmingen), KC/CXCL1 (rabbit anti-mouse GRO-α Ab; Abcam), and MIP-1α/CCL3 (goat anti-mouse MIP-1α; R&D Systems) or control Abs was performed. Primary Ab binding was detected with biotinylated Ab, avidin–biotin peroxidase complex, and 3,3’-diaminobenzidine substrate (VECTASTAIN Universal Quick Kit; Vector) or FITC-conjugated Ab. Slides were counterstained with hematoxylin. The neointimal area with specific immunostaining was determined using AnalySis software (Soft Imaging Systems, Münster, Germany) and expressed as percentage of the total neointimal area. To colocalize JE/CCL2 and platelet P-selectin in the same sections, double immunofluorescence staining was performed using the aforementioned primary Abs and a secondary FITC-conjugated Ab or the ABC-AP kit and fluorescent Vector Red substrate for detection.

Enzyme-Linked Immunosorbent Assay
Frozen carotid arteries and peripheral blood cells isolated by centrifugation were homogenized in GETP-buffer (20% glycerin, 1 mmol/L EDTA, 20 mmol/L Tris-HCl, 0.1 mmol/L PMSF). JE/CCL2, KC/CXCL1, and MIP1-α/CCL3 concentrations in serum or homogenates were determined using standard enzyme-linked immunosorbent assay protocols (R&D Systems) and normalized to total protein content (Biorad DC Protein Assay for homogenates). Primary murine SMCs isolated from apoE−/− carotid arteries, characterized by expression of SMC-specific genes and cultured in vitro, were stimulated with tumor necrosis factor (TNF)-α (200 U/mL; Pepro Tech) for 6 or 24 hours (n=3 each group). Total protein content and JE/CCL2 concentrations were determined in supernatants.

Flow Cytometry
Platelets from wild-type and CCR2−/− mice were isolated as described.18 Activated (thrombin receptor–activating peptide [TRAP] 2 μmol/L; Bachem) and unstimulated platelets (2×10⁷/mL) were incubated with biotinylated JE/CCL2 (0.5 to 1.5 μg/mL) or control protein and avidin-fluorescein (JE/CCL2 Biotinylated Fluorokine Kit; R&D Systems), and specific JE/CCL2-binding was analyzed by flow cytometry (FACSCalibur; BD Biosciences). Surface-bound JE/CCL2 on circulating platelets and leukocytes was analyzed by flow cytometry of peripheral blood drawn before (0 hours) and 6 hours after injury. Using two-color fluorescence, JE/CCL2 was detected by a PE-conjugated Ab (2H5; Pharmingen) on platelets and leukocytes identified by FITC-conjugated Abs to CD41 (MWREG38; Research Diagnostics) or CD11b (M1/70; Pharmingen) and gated accordingly. For the detection of surface JE/CCL2, murine platelets were incubated with conditioned media from murine arterial SMCs left unstimulated or stimulated with TNF-α (200 U/mL) for 6 hours or 24 hours, reacted with PE-conjugated JE/CCL2 Ab or control Ab, and analyzed by flow cytometry (n=3 each group). Surface CCL2 was also analyzed on resting and TNF-α–stimulated murine SMCs.

In Vitro Adhesion Assays
Flow chamber assays were performed as described.21 Human platelets were incubated with/without hCCL2 (0.5 μg/mL) and washed, stimulated with TRAP (15 μmol/L), and immobilized on sialinized glass slides. MM6 cells (10⁶/mL) pretreated with/without s-MCP-1 (1 μg/mL) were perfused over surface-adherent platelets at 0.75 dyne/cm² to analyze adhesion in multiple high-power fields. To study the shear-resistant arrest of monocytes on activated SMCs, cultured murine arterial SMCs were stimulated with TNF-α (200 U/mL) for 24 hours, and after preincubation of SMCs with the blocking CCL2 Ab 2H5 or control Ab, murine monocytes (10⁷/mL, WEHI-274.1 cells, ATCC) were perfused at 1.0 dyne/cm² (n=3 each group).

Statistical Analysis
Statistical analysis was performed using Prism 4.0 software (GraphPad). Data represent mean±SEM of 3 to 6 mice or experiments. Data were compared by unpaired, two-tailed t test with Welch correction or one-way ANOVA and Newman-Keuls post-test when appropriate. Differences with P<0.05 were considered as statistically significant.

Results
Localization of JE/CCL2 Expression
JE/CCL2 was not detected by immunohistochemistry in unjured carotid arteries of apoE−/− mice on standard chow (Figure 1A). After 1 week of Western-type diet, JE/CCL2 staining was evident in the media of carotid arteries (Figure 1B). Carotid sections from hyperlipidemic apoE−/− mice 24 hours after injury revealed intense staining for JE/CCL2 in all medial SMCs (Figure 1C). Notably, platelets adherent to the denuded vessel wall 24 hours after injury as identified by P-selectin immunostaining (Figure 1D) showed strongly positive staining for JE/CCL2 (Figure 1C, inset). Sections reacted with control antibody were negative (not shown). In contrast, MIP-1α/CCL3 was not detectable in the media or adherent platelets but was detectable in mononuclear cells infiltrating the adventitia (Figure 1E), whereas KC/CXCL1 was not expressed in the arterial wall 24 hours after wire injury (Figure 1F).

Colocalization of luminal JE/CCL2 with P-selectin on surface-adherent platelets covering the denuded arterial wall was confirmed by double immunofluorescence staining of carotid arteries 24 hours after wire injury (Figure 2).
Enzyme-Linked Immunosorbent Assay
Quantification of JE/CCL2 in apoE−/− Carotid Artery Tissue, Serum, and Blood Cells

Analysis of JE/CCL2 levels in homogenates of carotid arteries revealed a marked increase of JE/CCL2 content after wire injury compared with uninjured arteries, peaking at 24 hours (546 ± 105 versus 31 ± 15 fg JE/CCL2 per μg tissue protein; P < 0.001; n = 5) and declining at 48 hours (Figure 3A). In contrast, serum JE/CCL2 was transiently increased only at 6 hours after injury (177 ± 12 versus 116 ± 16 pg/mL; P < 0.01; n = 4) and declined at 24 hours and 48 hours to levels lower than in uninjured mice (Figure 3B). Significant amounts of CCL2 in the circulation are cell-associated.22 However, the JE/CCL2 content in homogenates of peripheral blood cells (176 ± 27 fg/μg protein before injury) was not significantly altered 6 hours and 24 hours after wire injury. By comparison, KC/CXCL1 was not detected in homogenates from carotid arteries (Figure 3C) or peripheral blood cells (not shown). Serum KC levels were increased 24 hours after wire injury and also after sham operation (not shown). MIP-1α/CCL3 content was slightly increased in carotid artery tissue 24 hours after injury (Figure 3C), consistent with the adventitial infiltrate, but not detected in serum or blood cell homogenates (not shown).

Binding of Recombinant and SMC-Derived JE/CCL2 to Platelets

Because platelets are known to contain numerous chemokines but not CCL2,23 the capability of platelets to bind JE/CCL2 was evaluated in vitro by flow cytometry. Binding of biotinylated JE/CCL2 to platelets was detected at 0.5 μg/mL (mean fluorescence intensity 269.7 ± 1.4 versus 62.6 ± 14.7 control; n = 3 to 9; P < 0.0001; Figure 4A), which substantially exceeds peak serum levels. Increasing concentrations up to 1.5 μg/mL enhanced JE/CCL2 binding in a linear fashion. Nonlinear regression analysis demonstrated that specific JE/CCL2 binding to platelets was not fully saturated at concentrations > 1.5 μg/mL, likely because of quenching of the fluorescent dye (Figure 4B). Platelet activation with TRAP or CCR2 deficiency did not alter JE/CCL2 binding (data not shown). As revealed by two-color flow cytometry circulating CD41+ platelets did not exhibit JE/CCL2 surface immobilization (Figure 4C and 4D), whereas JE/CCL2 binding to CD11b+ leukocytes was detected before and 6 hours after injury (Figure 4E and 4F). To study whether CCL2 derived from SMCs can bind to the platelet surface, vascular SMCs were stimulated in vitro with TNF-α to induce CCL2 expression and secretion (Figure 5A). Although CCL2 surface expression could not be detected on TNF-α–stimulated SMCs by flow cytometry (data not shown), the incubation of isolated platelets with conditioned medium from SMCs activated for 6 hours or 24 hours interestingly resulted in a time-dependent increase in CCL2 bound to the platelet surface (Figure 5B).

JE/CCL2 Mediates Monocyte Adhesion on Adherent Platelets and in Injured CCAs

Preincubation of platelets with hCCL2 resulted in enhanced arrest of mononuclear cells in flow, which was prevented by pretreatment with the CCR2 antagonist 76MCP-1 (Figure 6A). Although similar results were obtained using isolated human blood monocytes, CCL2 immobilized on glass slides in the absence of platelets or on dishes coated with fibronectin did not induce monocyte arrest (not shown). This indicates that CCL2 presented on adherent platelets triggers CCR2-dependent monocyte arrest in flow. In contrast, CCL2 was not involved in the arrest of monocytes on TNF-α–stimulated...
SMCs isolated from apoE<sup>−/−</sup> carotid arteries (Figure 6B), which produced high amounts of soluble CCL2 but did not express CCL2 on their surface. To explore whether early monocyte recruitment after endothelial denudation is dependent on JE/CCL2, ex vivo perfusion of CCAs isolated from apoE<sup>−/−</sup> mice with monocytic MM6 cells (Figure 7A) or murine EGFP<sup>+</sup> monocytes (Figure 7B) was performed 24 hours after wire injury. Monocytes rapidly accumulated mostly after a short distance of rolling on the luminal surface of the CCA (Figure 7A and 7B). After preperfusion with a blocking JE/CCL2 antibody, monocytes were still able to roll on the surface of the injured artery (Figure 7A,B), but firm arrest was reduced by >80% as compared with assay buffer or isotype control antibody (Figure 7C and 7D). Notably, preperfusion with blocking JE/CCL2 antibody did not inhibit monocyctic cell arrest on early atherosclerotic endothelium in the uninjured internal carotid artery (Figure 7A).

**Effect of CCR2 Deficiency on Neointimal Formation in apoE<sup>−/−</sup> Mice**

To evaluate the role of CCR2 after arterial injury in hyperlipidemic mice, we performed carotid injury in CCR2<sup>−/−</sup>/apoE<sup>−/−</sup> and CCR2<sup>+/+</sup>/apoE<sup>−/−</sup> mice. Total cholesterol and triglyceride levels did not differ between both groups (not shown). Neointimal area was reduced by 47% in CCR2<sup>−/−</sup>/apoE<sup>−/−</sup> compared with CCR2<sup>+/+</sup>/apoE<sup>−/−</sup> mice 28 days after injury (Figure 8A to 8C). Analysis of the cellular composition in neointimal lesions revealed a 79% reduction in the relative content of Mac-2–positive macrophages in CCR2<sup>−/−</sup>/apoE<sup>−/−</sup> mice (Figure 8D to 8F). Conversely, the neointimal SMC content was increased in
CCR2<sup>−/−</sup>/apoE<sup>−/−</sup> mice (Figure 8G to 8I). Similarly, neointimal collagen I content was expanded in CCR2<sup>−/−</sup>/apoE<sup>−/−</sup> mice (22.0 ± 1.8% versus 16.0 ± 1.8%; n = 5; P < 0.05). Thus, CCR2 deficiency results in a more stable plaque phenotype.

Discussion

In a hyperlipidemic mouse model of acute vascular injury, we found a rapid upregulation of JE/CCL2 in medial SMCs after endothelial denudation, a transient increase in serum levels and retention of secreted JE/CCL2 in platelets and triggered monocyte arrest in adherent platelets. Accordingly, JE/CCL2 mediated early monocyte arrest in denuded and ex vivo perfused carotid arteries. Deficiency of CCR2 in apoE<sup>−/−</sup> mice was accompanied by a reduction of neointimal area and monocyte infiltration but increased SMC content.

Recent studies provide evidence that platelets are involved in monocyte recruitment to early atherosclerotic and neointimal lesions by secretion and deposition of chemokines. It has been found that platelets contain JE/CCL2, which is released upon activation and binds to monocytes. The interaction between JE/CCL2 and CCR2 on monocytes is crucial for monocyte recruitment to atherosclerotic plaques. In addition, platelets can be activated by chemokines, such as JE/CCL2, which promotes adhesion and aggregation of platelets, contributing to the formation of atherosclerotic plaques. The role of platelets in monocyte recruitment is further supported by experiments showing that platelet depletion reduces monocyte infiltration into atherosclerotic lesions.

CCL2<sup>−/−</sup> mice showed strong immunoreactivity for JE/CCL2 in colocalization with platelet P-selectin, which was specific and not observed with other chemokines involved in monocyte recruitment, such as KC or MIP-1α. Our in vitro studies confirmed specific and CCR2-independent binding of JE/CCL2 to human platelets with low affinity. Surface binding may be mediated by glycosaminoglycans, as described for endogenous platelet chemokines, such as PF4. Serum levels of JE/CCL2 were transiently increased after wire injury but negligible when compared with concentrations required for binding to platelet suspensions in vitro. Moreover, CD11b<sup>+</sup> leukocytes but not platelets in the circulation displayed significant JE/CCL2 surface binding before and after injury. The protein-adjusted JE/CCL2 content in the arterial wall was profoundly increased 24 hours after injury and exceeded levels asso-
associated with circulating blood cells. In line with the notion that high levels of JE/CCL2 at the injury site are sufficient for immobilization on platelets covering the arterial wound area, whereas peripheral levels are too low to yield surface binding to circulating platelets, we propose that JE/CCL2 presented on adherent platelets at the site of denudation is mainly derived from vascular rather than peripheral sources. Interestingly, activated platelets can induce CCL2 secretion from cultured SMCs. Thus, the close vicinity of platelets to CCL2-secreting SMCs may not only limit the elution of CCL2 to peripheral blood by surface binding of CCL2 but may also provide timely amplification of local CCL2 production at sites of arterial injury where platelet adhesion and contact with SMCs occur.

Monocyte adhesion on inflamed endothelium in flow depends on surface-immobilized GRO-α/CXCL1 and CXCR2 but not MCP-1/CCL2 and CCR2. Correspondingly, monocyte accumulation on early atherosclerotic endothelium in carotid arteries of apoE<sup>−/−</sup> mice perfused ex vivo is mediated by the murine GRO-α ortholog KC and CXCR2 but not CCL2/CCR2, although both chemokines are expressed in the vessel wall. Our findings that blockade of JE/CCL2 reduced monocyte arrest in injured carotid arteries of apoE<sup>−/−</sup> mice, whereas its addition increased monocyte arrest on adherent platelets implies a distinctive contribution to monocyte recruitment after endothelial denudation. Using a blocking CCL2 antibody, the differential involvement of CCL2 in monocyte arrest in denuded segments but not on atherosclerotic endothelium of uninjured segments was illustrated side-by-side in the same artery. This difference in arrest function is likely attributable to the local concentration and presentation of JE/CCL2 by platelets adherent to denuded arteries, a mechanism absent in uninjured arteries. Notably, CCL2 expression is upregulated in neointimal SMCs but secretion of CCL2 does not result in surface immobilization on SMCs. This may explain why the increase in monocyte arrest on cytokine-activated SMCs or neointimal SMCs under flow is independent of CCL2.

Early P-selectin–mediated leukocyte recruitment to injured arteries is considered a key event in neointima formation after wire injury. Administration of a blocking...
CCL2 antibody within 24 hours after wire injury revealed critical role of CCL2 in this process. Accordingly, deficiency of CCR2 markedly reduced neointimal hyperplasia and macrophage content in hypercholesterolemic apoE−/− mice, suggesting a causal relationship between initial JE/CCL2-dependent monocyte arrest on denuded vessels and subsequent neointima formation. Notably, we and others found that the relative content of neointimal SMCs after vascular injury was increased in a context of hypercholesterolemia. This contrasts normocholesterolemic models in which neutralization of CCL2 reduced neointimal SMC but not macrophage content. Based on our data, we hypothesize that JE/CCL2 expression by medial SMCs after wire injury may be aggravated in hypercholesterolemia, resulting in presentation of sufficient JE/CCL2 concentrations on adherent platelets to preferentially trigger monocyte recruitment. Conversely, the neointimal expansion of SMCs in CCR2−/−/apoE−/− mice may be attributable to a shift in the complex interplay of plaque chemokines toward increased activity of stabilizing chemokines, such as SDF-1α, implicated in neointimal recruitment of SMC progenitors.

In summary, our data define a novel function of JE/CCL2 as an arrest chemokine in early monocyte recruit-
ment after endothelial denudation of arteries in hyperlipidemic mice, which appears to require its local concentration and presentation on adherent platelets. This may represent the principle mechanism accounting for reduced neointimal hyperplasia and macrophage content in CCR2-deficient apoE−/− mice.

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Platelet-Bound CCL2 in Neointimal Hyperplasia

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The crucial role of the CCL2/CCR2 axis in neointimal hyperplasia after arterial injury in hyperlipidemic mice involves early monocyte recruitment and CCL2 presentation on platelets

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Supplemental Figure: This scheme depicts the areas of the carotid artery analyzed for rolling and adhesion of monocytes after wire-injury in ex-vivo perfusion experiments. Endothelial denudation was performed by retrograde insertion of an angioplasty guide-wire through the external carotid artery (ECA) into the common carotid artery (CCA). The internal carotid artery (ICA) was not denuded by this approach. Since the ECA was occluded after wire insertion, the injured ECA area was omitted from the analysis because of the absence of physiological blood flow. The uninjured ICA and injured ECA were delineated by two imaginary perpendicular axes which were dropped through the origin of the ICA and ECA at the bifurcation.