CX3CR1 Deficiency Confers Protection From Intimal Hyperplasia After Arterial Injury

Peng Liu, Sarita Patil, Mauricio Rojas, Alan M. Fong, Susan S. Smyth, Dhavalkumar D. Patel

Objective—A functional polymorphism in the chemokine receptor CX3CR1 is associated with protection from vascular diseases including coronary artery disease and internal carotid artery occlusive disease. We investigated the mechanisms by which CX3CR1 may be involved by evaluating the inflammatory response to arterial injury in CX3CR1-deficient animals.

Methods and Results—Femoral arteries of CX3CR1−/− and wild-type (WT) mice were injured with an angioplasty guide wire. After 1, 5, 14, and 28 days, arteries were harvested and evaluated by histology, morphometry, and immunohistochemistry. Arterial injury upregulated the CX3CR1 ligand CX3CL1. In CX3CR1−/− compared with WT animals, the incidence of neointima formation was 58% lower (P=0.0017), accompanied by no difference in the area of platelet accumulation at day 1 (P=0.48) but a significant decrease in intimal monocyte infiltration at day 5 (P=0.006), vascular smooth muscle cell (VSMC) proliferation at days 5 and 14, and intimal area at day 28 (P=0.009).

Conclusions—In an endothelial denudation injury model, CX3CR1 deficiency protects animals from developing intimal hyperplasia as a result of decreased monocyte trafficking to the lesion. CX3CR1 deficiency decreases VSMC proliferation and intimal accumulation either directly or indirectly as a result of a defective monocyte infiltration. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words:
CX, CR1 in the development of intimal hyperplasia. A deficiency in CX, CR1 does not affect platelet function, but does significantly influence monocyte recruitment. CX, CR1 deficiency also plays a role in regulating SMC activities.

Materials and Methods

Animal Care
CX, CR1−/− (KO) mice were backcrossed onto the C57BL/6J background for 12 generations, and age-matched CX, CR1+/− (WT) mice generated from littermates were used as controls. All mice were bred and maintained in the barrier facility at the University of North Carolina and fed Prolab RMH-3000 (PMI Nutrition International, Richmond, Ind), a normal rodent chow diet. All experimental protocols were in compliance with IACUC guidelines and were approved by the IACUC at the University of North Carolina at Chapel Hill.

Femoral Artery Injury
Femoral arteries of 8- to 12-week-old male CX, CR1−/− and WT mice were injured by endoluminal passage of an angioplasty guide wire as previously described.22 Arteries on one side were not injured and served as negative controls. Briefly, mice were anesthetized with inhaled isoflurane and femoral arteries were exposed by a longitudinal groin incision and viewed under a surgical microscope. The distal portion of the artery was encircled with an 8-0 nylon suture, a vascular clamp was placed proximally at the level of the inguinal ligament, and a 0.01-inch diameter guide wire (CrossIT-200XT; Guidant Corporation, Indianapolis, Ind) was introduced into the arterial lumen through an arteriotomy made in the distal perforating branch. After release of the clamp, the guidewire was advanced to the level of the aortic bifurcation and immediately pulled back 3 times to denude the endothelium. After removal of the wire, the arteriotomy site was ligated and skin was closed. Animals were routinely monitored after surgery.

Tissue Preparation, Histology, and Morphometry
Mice were euthanized at days 1 (n = 20), 5 (n = 16), 14 (n = 8), and 28 (n = 26) after arterial injury. Animals were perfused with phosphate-buffered saline for 5 minutes, followed by 4% paraformaldehyde for 20 more minutes at 100 cm H2O via cannulation of the left ventricle. The hind limbs were then harvested en bloc, fixed in 4% paraformaldehyde overnight, and decalified in formic acid bone decalifier (Immunocal; Decal Corporation, Tallman, NY) for 24 hours. Tissues containing the femoral artery were embedded in paraffin and cut into 5-μm sections for further analysis.

Six to 10 sections per femoral artery at 100-μm intervals were screened with H&E staining, and sections from the area with maximal injury response were further evaluated by staining with the Combined Mason’s elastin (CME) stain to visualize the arterial wall layers or processed for immunohistochemistry. The intima and media areas were measured by computerized morphometry (Image J, National Institutes of Health). Intimal hyperplasia was defined as the formation of a neointimal layer within the internal elastic lamina (IEL). Media area was calculated as the area encircled by the external elastic lamina (EEL) minus the area encircled by IEL. The intima-to-media (IM) ratio was calculated as the intimal area divided by the media area. Arteries with a broken IEL or thrombosis by CME stain were excluded from the study.

Immunochemistry
For characterizing the molecular and cellular composition of arteries, immunohistochemical analysis was used to identify monocytes by mAb F4/80 (Serotec Inc, Raleigh, NC), VSMCs by alkaline phosphatase conjugated anti-α-actin (Sigma, St. Louis, Mo), platelets by anti-thrombocyte (Inter-Cell Technologies, Princeton, NJ), endothelial cells by anti-von Willebrand factor (vWF) (DakoCytomation Inc, Carpinteria, Calif), and CX,CL1 by anti-fractalkine (R&D Systems, Minneapolis, Minn). Recombinant mouse fractalkine/CX,CL1 (R&D Systems) was used to validate the specificity of CX,CL1 staining. Antigen retrieval was performed for F4/80 staining with trypsin digestion and for CX,CL1 and vWF staining by steaming in a citrate buffer (0.01 mol/L, pH 6.0) for 40 minutes. Endogenous peroxide activity was quenched using 3% H2O2 in methanol for 10 minutes at room temperature, and sections were blocked using 4% serum (goat, rabbit, or rat) for 10 to 60 minutes at room temperature. Primary antibodies and their respective controls were incubated overnight at 4°C. After washing, sections were incubated with a species-specific biotinylated secondary antibody (anti-mouse, anti-rabbit, anti-rat, and anti-goat, 1:200; Vector Laboratories, Burlingame, Calif) for 60 minutes at room temperature, followed by washes, and a subsequent 60-minute incubation at room temperature with streptavidin-horseradish peroxidase (Peroxidase Vectastain ABC Kit) to amplify antibody signal. After another wash, 3’, 3’-diaminobenzidine (DAB) (Sigma, St. Louis, Mo) was used as a substrate for the peroxidase. Alkaline phosphatase staining by α-actin antibody was visualized with the Vector Red Alkaline Phosphatase Substrate Kit (Vector Laboratories).

VSMC Proliferation Analysis
Four days after arterial injury, mice were injected intraperitoneally with three doses of bromodeoxyuridine (BrdU) (Roche Diagnostics, Basel, Switzerland) of 30 mg/kg at 8-hour intervals before euthanasia. Arteries were harvested on day 5 after injury, and proliferating cells were identified by immunostaining with an anti-BrdU antibody (Roche Diagnostics). For all times other than day 5 after injury, an anti-PCNA antibody (Santa Cruz Laboratories, Santa Cruz, Calif) was used to identify proliferating cells. Proliferating VSMCs were defined as α-actin-positive cells that were also positive for BrdU or PCNA staining in series sections. The proliferation index was calculated as the percentage of BrdU-stained or PCNA-stained nuclei of the total number of nuclei in the indicated area. VSMC proliferation was also determined in vitro utilizing primary cultures of VSMCs isolated from aortas of CX,CR1−/− and WT mice with a colorimetric assay based on the uptake of MTT by viable cells (Cell Proliferation Kit; Roche Applied Science, Indianapolis, Ind).

Platelet Function
Platelet function was tested in vitro with the Cone and Plate(let) Analyzer (CPL) system using a DiaMed Impact-R machine (DiaMed Israel Ltd) according to the manufacture’s instructions. Briefly, fresh samples of sodium citrated anticoagulated blood (130 μL) from CX, CR1, and WT mice were placed in polystyrene wells and subjected to defined shear (1800/s) for 2 minutes. The samples were then thoroughly washed with phosphate-buffered saline, stained with May-Grünwald stain, and quantitated with an image analyzer. Platelet deposition on the polystyrene surface was evaluated by examining: (1) the percentage of total area covered with platelets designated as surface coverage; and (2) average size in μm2 of surface-bound platelet thrombi.

Monocyte Adhesion
Monocyte adhesion was evaluated as previously described.14 Briefly, spleens from CX,CR1−/− and WT mice were homogenized in RPMI-1640 with 10 mmol/L HEPES using a manual tissue homogenizer. Red blood cells were lysed with red blood cell lysis buffer (0.14 mol/L NH4Cl, 0.017 mol/L Tris-HCl pH 7.5, adjust to pH 7.2). Cells were washed with RPMI-1640 with 10% fetal bovine serum (FBS), passed through a 70-μm nylon filter, and suspended in RPMI-1640 with 10% FBS. Adhesion of the splenocytes to fractalkine under physiological flow conditions was then tested by the parallel plate flow chamber assay with recombinant fractalkine-secreted alkaline phosphatase fusion proteins immobilized on a glass coverslip. After the run, adherent cells were stained with MOMA-fluorescein isothiocyanate (FITC) antibody (Beckman Coulter, Inc, Fullerton, Calif) and the number of monocytes bound was quantified by counting MOMA+ cells.
CX₃CR1 Expression Is Induced in Injured Arteries

To define the role of CX₃CR1 in the response to arterial injury, we first examined the expression pattern of its ligand, CX₃CL1, by immunohistochemistry. CX₃CL1 expression was undetectable in non-injured, control arteries at any stage of the injury response. In injured arteries, CX₃CL1 was not continuously detected between 5 and 28 days after injury. These data suggest that CX₃CL1 may be involved in the pathogenesis of guide wire induced vascular injury.

CX₃CR1-Deficient Mice Are Protected From Intimal Hyperplasia

To assess whether CX₃CR1 plays a role in mediating intimal hyperplasia after arterial injury, we measured the incidence and extent of neointima formation in CX₃CR1⁻⁻ mice and WT mice. The overall incidence of intimal hyperplasia at 5, 14, and 28 days was decreased in CX₃CR1⁻⁻ mice by 58% (8/21 KO versus 26/29 WT, P=0.0017). Compared with the average intimal area in WT mice, the average intimal area in CX₃CR1⁻⁻ mice was reduced by 84%, 56%, and 74% at 5, 14, and 28 days, respectively (Figure 3). In contrast, no significant differences in luminal and medial areas were detected between WT and CX₃CR1⁻⁻ arteries (data not shown). These data indicate that CX₃CR1 deficient mice are protected from intimal hyperplasia after acute arterial injury, and that the arterial injury model is appropriate to study the functional roles of CX₃CR1.

Normal Platelet Function in CX₃CR1⁻⁻ Mice

To define whether platelet CX₃CR1 is functionally relevant in guide wire induced injury, we examined platelet accumulation on the injured luminal surface by immunostaining. By morphometric analysis, there was no difference in the area of the platelet thrombus that accumulated along injured CX₃CR1⁻⁻ and WT arteries (Figure 4A and 4B). We also examined platelet function under near-physiological conditions in vitro using blood collected from CX₃CR1⁻⁻ and WT mice. As shown in Figure 4C and 4D, the absence of CX₃CR1 did not affect shear-induced platelet thrombus formation. These data suggest that platelet CX₃CR1 may be not an essential mediator of platelet adhesion or aggregation in response to arterial injury or high shear.

Defective Monocyte Recruitment in CX₃CR1⁻⁻ Mice

CX₃CR1 is believed to be critically important for monocyte recruitment to the inflamed or injured vessel. We tested this hypothesis by examining monocyte infiltration into the vascular wall by F4/80 antigen staining (Figure 5A). There was a 100% and an 87% decrease in monocyte accumulation in CX₃CR1⁻⁻ mice at 5 and 14 days, respectively, compared with WT mice (Figure 5B). Monocyte adhesion to CX3CL1
under physiological flow conditions was also substantially lower with CX3CR1−/− compared with WT cells (Figure 5C). Taken together, these results provide strong evidence that CX3CR1 plays a critical role in monocyte recruitment to the inflamed vessel wall.

**Role of CX3CR1 in VSMC Response to Vascular Injury**

To assess whether CX3CR1 is important for the function of VSMC in response to arterial injury, we took 3 approaches. First, we evaluated the numbers of VSMC in the vessel wall by immunohistochemistry for α-actin. Second, we evaluated VSMC proliferation by BrdU incorporation and by immunohistochemistry for PCNA expression in α-actin positive cells. Third, we evaluated the in vitro proliferation of primary VSMC isolated from CX3CR1−/− and WT mouse aortas.

In injured arteries, substantial VSMC proliferation was observed at 5 days and 14 days (Figure 6). At 5 days, most proliferating cells were localized in the media. The proliferation index in CX3CR1−/− mice was decreased by 45% compared with WT mice (8.5±2.7% versus 15.3±3.4%; P=0.07). As the lesion progressed at 14 days, an 85% decrease in proliferating cells was detected in the intima of CX3CR1−/− mice compared with WT animals (3.1±2.0% versus 20±6.0%; P=0.03). In vitro, while WT aortic VSMC proliferated in response to CX3CL1 (P=0.005), CX3CR1−/− aortic VSMC did not (P=NS). These results suggest that CX3CR1 deficiency diminishes VSMC proliferation in the early development of intimal hyperplasia in response to arterial injury.

**Discussion**

Inflammation is a driving force behind vascular diseases such as atherosclerosis and restenosis. Arterial injury is the initial stage of the pathohistological changes of these diseases. We found that guidewire-induced endothelial denudation of mouse femoral arteries elicits an inflammatory response with upregulation of CX3CL1 expression. Therefore, we investigated the mechanisms of action of CX3CR1 in vascular inflammation by testing the effects of CX3CR1 deficiency in this injury model, which stimulates acute inflammatory responses similar to restenosis. Because CX3CR1 is expressed on platelets, monocytes and VSMC, we focused on these cell types.

CX3CR1 does not appear to play a critical role in platelet accumulation along the denuded endothelial surface. In our

**Figure 3.** Injury-induced intimal hyperplasia in wild type (WT) and CX3CR1−/− (KO) mice. Unilateral wire injury was performed in femoral arteries of WT and CX3CR1−/− mice, with the contralateral uninjured side used as a negative control. A, CME stains of representative sections of control and injured femoral arteries at 28 days after injury. Intimal hyperplasia is defined as the formation of a neo-intimal layer within the internal elastic lamina (arrows). B, Average intimal areas of injured WT and KO arteries at 5, 14, and 28 days after injury. Results are reported as mean±SEM. "P<0.05.
model, platelets and neutrophils cover the denuded luminal surface within 24 hours.22–24 The area of platelet accumulation was unaffected by the absence of CX3CR1. Likewise, no significant differences were observed in shear-induced platelet thrombus formation in blood from WT and CX3CR1-deficient mice. These results suggest that platelet CX3CR1 does not play a major role in the initial phases of platelet adhesion and aggregation stimulated by the vascular injury. Likewise, CX3CL1 was not highly expressed within the first 24 hours after injury. Whether CX3CR1 contributes to other platelet responses cannot be determined from our study.

Regenerated endothelial cells expressed high levels of CX3CL1. Similar to previous reports,22 we observed regeneration of endothelial within 5 days of arterial injury. The endothelium before injury did not express CX3CL1, but the regenerated endothelium expressed high levels of CX3CL1. In contrast to WT animals that had robust monocyte accumulation, CX3CR1−/− mice failed to recruit monocytes to the intima despite expression of CX3CL1. Interestingly, monocytes accumulated in the adventitia of WT and CX3CR1−/− animals after injury, suggesting that monocyte recruitment to the adventitia may occur through mechanisms distinct from those required for intimal recruitment. A primary difference between these two sites is the presence of arterial shear forces, and monocyte capture and transmigration along the artery may require CX3CR1-driven adhesion and/or migration to resist the shear. In contrast, recruitment of tissue macrophages or transvenous migration of monocyte to the adventitia may occur by CX3CR1-independent mechanisms. While we cannot exclude the possibility that adventitial macrophages traffic and migrate toward soluble CX3CL1 through the vessel wall to the luminal surface, the data support a primary role for CX3CR1 in the rapid capture and firm adhesion of monocytes under flow conditions. Clinical cohorts have shown that 2 single nucleotide polymorphisms of CX3CR1, V249I and T280, are associated with reduced prevalence of atherosclerosis and coronary artery disease.8,9 In addition, the M280 allele is associated with a reduced risk of internal carotid artery (ICA) occlusive disease.10 Our laboratory has shown that the protein encoded by the M280 allele has impaired adhesive capacity,7 suggesting that cell adhesion is an important mechanism by which CX3CR1 exerts its effect on recruiting cells during vascular inflammation.

In addition to CX3CR1, CCR2 appears to mediate the response to arterial injury as well. In the same mouse model of wire-induced femoral artery injury, CCR2−/− mice had a phenotype comparable to CX3CR1−/− mice in terms of a reduced intimal hyperplasia and intima/media ratio 4 weeks after injury.23 In the CCR2 study, no macrophage infiltration was seen by MOMA-2 or CD68 staining in either WT or CCR2−/− animals and the authors concluded that CCR2 did not affect macrophage accumulation within the arterial wall after injury. Although both CX3CR1 and CCR2 mediate
monocyte chemotaxis and trafficking to sites of inflammation, CXCR1 mediates direct monocyte binding to its membrane-bound ligand, CX3CL1, without the help of other adhesion molecules, such as integrins.15 Thus, monocyte trafficking to damaged tissue during arterial injury may favor the CX3CR1–CX3CL1 system. Recently, Schober et al reported an alternative mechanism for CCR2 in acute vascular injury.24 In their study, CCL2 was found to be co-localized with platelets on the denuded carotid artery surface of apoE−/− mice fed with a high-fat diet within 24 hours after wire injury. Even though platelets do not contain CCL2,26 low-affinity CCR2-dependent binding of CCL2 to human platelets is detected in vitro,27 suggesting that adherent platelets with immobilized CCL2 mediate monocyte recruitment after mechanical injury. Monocyte adhesion to the luminal surface is not detected within 24 hours under our experimental conditions or in similar wire-induced arterial injury models under either normolipidemic23 or hyperlipidemic25,28 conditions. Nevertheless, platelet deposition may play a vital role in the pathogenesis of restenosis,29 and CCR2 may be involved in that process.

SMC accumulate in the intima in both atherosclerosis and restenosis.30,31 This process is also seen in animals following guidewire-induced arterial injury. CXCR1 is expressed in human SMC cultured from coronary arteries and in atherosclerotic lesions,17,18 suggesting that CXCR1 may play a role in regulating SMC function. In our study, intimal hyperplasia was significantly decreased in CXCR1−/− arteries after injury. Concurrently, there were decreased numbers of VSMC in the neointima. VSMC proliferation in response to injury was also impaired in CXCR1−/− mice as measured by both BrdU incorporation and PCNA immunohistochemistry. Whether the VSMC effects are primary or secondary cannot be addressed by this study. However, several data point to a primary effect of CXCR1 on VSMC function in this model. In a recent report of interleukin (IL)-15’s effect on CXCR1/CX3CL1 expression and the response to arterial injury,32 IL-15 attenuated SMC proliferation and CXCR1/CX3CL1 mRNA expression on serum stimulation. Using a periodontal injury model, the authors further demonstrated that IL-15 up-regulation of CX3CL1 expression protects humans from atherosclerotic diseases, our findings suggest that CXCR1 could be an effective drug target for both acute and chronic vascular injuries, such as restenosis and atherosclerosis.

### Acknowledgments

The authors sincerely thank Gail Grossman and Kirk McNaughton for their technical assistance in tissue processing and staining, Rishi Rampersad for animal husbandry and Drs Teresa Tarrant and Maya Jerath for proofreading the manuscript.

### Sources of Funding

This study was supported by National Institute of Health R01s CA098110 (D.D.P.) and HL074219 (S.S.S.).

### Disclosures

None.

### References


CX3CR1 Deficiency Confers Protection From Intimal Hyperplasia After Arterial Injury
Peng Liu, Sarita Patil, Mauricio Rojas, Alan M. Fong, Susan S. Smyth and Dhavalkumar D. Patel

Arterioscler Thromb Vasc Biol. published online June 29, 2006;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2006/06/29/01.ATV.0000234947.47788.8c.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/