Blockade of Keratinocyte-Derived Chemokine Inhibits Endothelial Recovery and Enhances Plaque Formation After Arterial Injury in ApoE-Deficient Mice

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**Objective**—We evaluated the involvement of keratinocyte-derived chemokine (KC) in neointimal hyperplasia and endothelial repair after arterial injury.

**Methods and Results**—Expression of KC was detected by immunohistochemistry in carotid arteries of apolipoprotein E−deficient (apoE−/−) mice not earlier than 2 weeks after wire-injury. Double immunofluorescence staining revealed a colocalization of KC with Mac-2–positive macrophages. Immunoreactivity for KC and its receptor CXCR2 was detectable in regenerating CD31-positive endothelial cells. Treatment of apoE−/− mice with a blocking monoclonal antibody (mAb) to KC after carotid injury for 3 weeks substantially increased neointimal plaque area compared with isotype control-treated or untreated mice. As assessed by luminal CD31 or VE-cadherin and Evans blue staining, neutralization of KC inhibited endothelial recovery in injured arteries, whereas macrophage and smooth muscle cell content were unaffected. In vitro, treatment with KC mAb, a blocking CXCR2 mAb, or the CXCR2 antagonist showed delayed KC-mediated endothelial cell chemotaxis and wound repair of endothelial monolayers after scratch injury. Conversely, addition of exogenous KC accelerated wound repair in a CXCR2-dependent manner.

**Conclusions**—Neutralization of KC increased plaque formation and delayed endothelial recovery after arterial injury, without affecting neointimal monocyte infiltration. As an underlying mechanism, KC was involved in promoting CXCR2-mediated endothelial chemotaxis and wound repair. *(Arterioscler Thromb Vasc Biol. 2004;24:1891-1896.)*

**Key Words:** atherosclerosis ■ chemokine ■ restenosis ■ reendothelialization ■ injury

Restenosis after acute arterial injury represents an accelerated form of atherosclerosis attributable to reactive constriction of the arterial wall and neointimal thickening. Initially, intraluminal injury results in endothelial denudation and platelet adhesion. Platelet-derived mediators and proinflammatory cytokines induce an inflammatory reaction triggering leukocyte recruitment to the injury site. Monocyte-derived macrophages persist in the developing neointima and provide an additional source for growth factors, whereas smooth muscle cells (SMCs) comprise a major part of the neointimal tissue stabilizing the injured vessel wall. Finally, reendothelialization contributes to the termination of neointimal growth.

Chemokines have been found to play a major role in neointimal hyperplasia after acute arterial injury. Numerous studies have elucidated the contribution of monocyte chemotactant protein-1 to the progression of neointimal growth, which affects monocyte or SMC content depending on the presence of hyperlipidemia. Moreover, RANTES secreted by activated platelets has been involved in neointima formation by promoting monocyte recruitment, whereas blocking stromal cell-derived factor (SDF)-1α inhibited neointima formation by reducing SMC content.

Although keratinocyte-derived chemokine (KC), the mouse ortholog of human growth-related oncogene (GRO-α), is essential for monocyte arrest on early atherosclerotic endothelium in mice, its role in acute vascular injury remains elusive. Therefore, we examined the effect of a blocking KC monoclonal antibody (mAb) on neointima formation after carotid wire-injury in apolipoprotein E−deficient (apoE−/−) mice.

**Methods**

**Atherogenic Murine Model of Restenosis**

Female 8-week-old apoE−/− mice (C57BL/6, M&B, Ry, Denmark) were fed an atherogenic diet, and carotid artery injury was performed using a coated guide wire as described. The mice (n=6 each group) were left untreated or were treated through IP injection with KC mAb (50 μg every 72 hours; clone 124014, R&D Systems) or isotype control. To ensure effective mAb concentrations, rat mAb content were unaffected. In vitro, treatment with KC mAb, a blocking CXCR2 mAb, or the CXCR2 antagonist showed delayed KC-mediated endothelial cell chemotaxis and wound repair of endothelial monolayers after scratch injury. Conversely, addition of exogenous KC accelerated wound repair in a CXCR2-dependent manner.

**Conclusions**—Neutralization of KC increased plaque formation and delayed endothelial recovery after arterial injury, without affecting neointimal monocyte infiltration. As an underlying mechanism, KC was involved in promoting CXCR2-mediated endothelial chemotaxis and wound repair. *(Arterioscler Thromb Vasc Biol. 2004;24:1891-1896.)*

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and carotid arteries were excised and paraffin-embedded. Animal experiments were approved by local authorities and complied with German animal protection law.

**Histomorphometry**
Serial sections (5 μm) within 1000 μm from the bifurcation (16 per mouse) of the left and right carotid arteries were stained with Movat’s pentachrome.6,7 The lumen and external elastic lamina areas were determined by computer-assisted planimetry (Diskus software, Hilgers), and overall vessel wall area was calculated as the difference. Because the coated wire generally caused a marked injury disrupting the internal elastic lamina, total vessel wall area was used instead of neointimal area. Arterial size was measured by tracing the external elastic lamina circumference.

**Quantitative Immunohistochemistry and Immunofluorescence**
Macrophage and SMC plaque content, reendothelialization, and KC expression were determined in serial sections within 1000 μm from the bifurcation (11 per mouse, 100 μm apart). Sections were stained for macrophages using Mac-2 mAb (clone M3/38, Cedarlane) for SMCs using α-actin mAb (clone 1A4, Dako) and for endothelial cells using CD31 mAb (clone M20, Santa Cruz) or VE-Cadherin (goat polyclonal, Santa Cruz). In addition, colocalization of KC (polyclonal rabbit GRO-α Ab, Abcam) or CXCR2 (mouse monoclonal, R&D Systems) with Mac-2 CD31 was performed by double immunofluorescence staining. To detect specific Ab binding, a secondary fluorescein isothiocyanate–conjugated Ab or the Vectafluor–conjugated Ab (all Vector Laboratories). Areas with specific immunostaining were determined by comparison to isotype controls and expressed as percentage of the total vessel wall area. Reendothelialization of the luminal surface was expressed as percentage of CD31-positive or VE cadherin–positive luminal lining of the total luminal circumference. ELISA was performed from serum samples using DuoSet ELISA Development System kit for mouse KC (R&D Systems).

**Evans Blue Staining**
Endothelial recovery was evaluated 3 weeks after wire-injury by staining the denuded areas with Evans blue dye (Sigma) as reported.10,11 Mice (n=3 each group) were treated as stated above. Briefly, 200 μL of saline containing 1% Evans blue was injected intracardially 10 minutes before euthanasia, followed by perfusion fixation with formalin for 5 minutes. The uninjured right and the injured left common carotid artery, including the adjacent portion of the bifurcation, were opened longitudinally and placed en face between microscopic slides. The total luminal area and area stained in blue were determined (Diskus software, Hilgers), and staining of the remaining endothelial defect was expressed as the percentage of the total surface (% area).

**Endothelial Wound Injury Repair Assay**
Simian virus 40–immortalized murine endothelial cells (SVEC, provided by Dr H. Hengel, Robert Koch Institute, Berlin, Germany) were grown to confluence using DMEM (containing 5% FCS) medium. KC concentrations were determined in supernatants using the DuoSet ELISA Development System kit (R&D Systems). Total RNA was extracted from cultured murine endothelial cells, and the expression of CXCR2 mRNA was detected using CytoXpress Multiplex–polymerase chain reaction (PCR) Kit (Biosource Interna-tional). Wounds were inflicted by dragging a sterile pipette tip across the monolayers creating a 1-mm cell-free path.11 Recombinant KC (100 ng/mL, Peprotech), KC mAb (10 μg/mL, clone 124014, R&D Systems), or GRO-α (25 μg/mL),11,12 or a blocking CXCR2 mAb (10 μg/mL, clone 48311, R&D Systems) or anti–vascular endothelial growth factor (VEGF) Ab (10 μg/mL, goat polyclonal, Sigma) were added for 8 hours after injury. Using phase contrast microscopy, the number of cells migrating into the wound area was counted and expressed as cells per mm.

**SVEC Proliferation Assay**
Cells were seeded in 96-well plate, incubated with 5-bromodeoxyuridine (BrdUrd) and treated with KC (100 ng/mL) with or without KC mAb (10 μg/mL), or GRO-α (25 μg/mL) or CXCR2 mAb (10 μg/mL) for 2 hours at 37°C (5% CO2). Cells were fixed with methanol, denatured, and incubated with a biotinylated BrdUrd Ab (Zymed BrdUrd Staining Kit, Zymed Laboratories). Streptavidin–fluorescein isothiocyanate was used as substrate, and absorbance was measured at 530 nm. As control, positive cells were counted by fluorescence microscopy and expressed as cells per field.

**Endothelial Transmigration Assay**
Transmigration assays with SVEC were performed as described.11 Briefly, Transwell inserts (5 μm pores; Costar) were coated with 0.2% gelatin for 30 minutes at 37°C. KC (100 ng/mL) was added to assay medium (DMEM/RPMI medium 1640, 0.5% bovine serum albumin) with or without KC mAb or isotype control. Cells were seeded into the top chamber (100 μL, 106 cells/mL) and allowed to transmigrate for 3 hours at 37°C. In the same experiments, SVECs were incubated with CXCR2 mAb, VEGF Ab, or isotype controls. Cells remaining in the top chamber after 3 hours were removed with a cotton swab. Migrated cells that adhered to the bottom surface of the filter were fixed and stained with 0.1% crystal violet in 0.1 mol/L borate, pH 9.0, and 2% ethanol. Stained cells were extracted with 10% acetic acid, and absorbance (optical density) at 620 nm was determined as a measure of transmigration.

**Statistical Analysis**
Data represent mean±SEM. Statistical analysis was performed with Prism 4 software (Graph Pad) using unpaired Student’s t test or 1-way ANOVA followed by Newman-Keuls post-hoc test. Differences with P<0.05 were considered significant.

**Results**
**KC Expression After Carotid Wire-Injury**
In carotid arteries of hyperlipidemic apoE−/− mice, KC expression was not detected by immunohistochemistry earlier than 2 weeks after injury (Figure 1A). Serum KC levels increased 6 hours after injury but declined to basal levels within 2 days (Figure 1B). This transient elevation was due to stress or dermal wounding, because it was also observed after sham-operation (Figure 1B). At later time points, KC serum levels slowly increased regardless of injury (Figure 1B), likely because of atherogenic diet.13 Combined immunofluorescence staining revealed a colocalization of KC with Mac-2–positive cells, confirming macrophages as an important source of KC in injured arteries (Figure 2A through 2C). Moreover, KC-specific staining was detected in CD31-positive endothelial cells covering the plaque (Figure 2D through 2F). Double immunofluorescence further demonstrated the presence of the KC receptor CXCR2 in regenerating CD31-positive cells after 3 weeks (Figure 2G through 2I).

**Effects of KC mAb on Plaque Formation**
To determine the functional implications of KC expression, neutralizing KC mAb or isotype control were administered after wire-injury in apoE−/− mice every 72 hours. Injection of KC mAb resulted in mAb serum levels sufficient to achieve sustained inhibition (1 to 15 μg/mL at 48 to 72 hours with an ND50 of 1 μg/mL) and in hardly detectable levels of KC after 3 weeks (data not shown). Treatment with mAb had no effect on total cholesterol and triglyceride levels (data not shown). Although the arterial size did not differ between KC mAb and isotype control-treated mice (1231±48 versus
1192 ± 19 μm), blockade of KC increased the total plaque area by 42% (89 287 ± 6905 versus 62 901 ± 5876 μm² for isotype control, n = 6, P < 0.05; Figure 3A through 3D). By contrast, total vessel wall area of the contralateral right uninjured carotid artery in the same mice (Figure 3A through 3C, inset) was unaltered by KC mAb (7464 ± 190 versus 8416 ± 616 μm² for isotype control). In addition, cellular plaque composition was not significantly affected as determined by the relative content of SMCs (36.2 ± 3.8% versus 33.9 ± 3.6% for isotype control) or of Mac-2–positive macrophages (17.8 ± 4.4% versus 12.3 ± 1.0% for isotype control) as a measure of leukocyte recruitment. Analysis of CD31 (Figure 3E through 3H) and VE-cadherin (Figure 3I through 3L) staining along the luminal surface of carotid arteries demonstrated that reendothelialization was far advanced but not entirely complete 3 weeks after injury in isotype control–treated or untreated mice, and that it was significantly reduced after treatment with KC mAb (Figure 3H and 3L). Staining of endothelial defects with Evans blue in injured arteries confirmed that endothelial recovery was almost complete 3 weeks after injury in isotype control–treated or untreated control mice (Figure 4A). Treatment with KC mAb resulted in a marked inhibition of endothelial recovery, as evident by the significant increase in the denuded area stained with Evans blue after 3 weeks (Figure 4B and 4C). The defects in the luminal surface appeared to comprise small and irregularly shaped areas, which may reflect the severity of the arterial injury and plaque formation obtained by using the coated wire.

**KC Contributes to Endothelial Wound Injury Repair In Vitro**

To address the role of KC in reendothelialization in detail, an in vitro model of endothelial wound repair was used. SVECs expressed KC (1.5 ± 0.3 ng/mL in supernatants as determined by ELISA, n = 4) and its receptor CXCR2 (Figure 5A). As KC was detected in both endothelial cells and plaque macrophages, the contribution of endothelial and exogenously added KC to wound repair was evaluated. Although the endothelial wound was almost completely closed after 8 hours, endothelial recovery was delayed in the presence of the KC mAb and appeared to be accelerated by addition of exogenous KC (Figure 5B through 5E). This hypothesis was confirmed by quantitative analysis: the number of cells that had migrated into the wounded area was significantly reduced by the KC mAb, a CXCR2 mAb, or the CXR2 antagonist 8-73 GRO-α but was significantly increased by exogenous KC (Figure 5F). The effect of exogenous KC was also inhibited by coinoculation with KC mAb, CXCR2 mAb, or 8-73 GRO-α, whereas isotype controls or a VEGF Ab had no effect (Figure 5F). Transwell-filter migration assays with SVEC clearly confirmed the ability of KC to induce endothelial cell chemotaxis (Figure I, available online at http://atvb.ahajournals.org). This effect was abolished by coinoculation with KC mAb or CXCR2 mAb but not by an Ab to VEGF (Figure I). In contrast, proliferation of SVEC was unaltered by KC, KC...
mAb, CXCR2 mAb, or α1,2 GRO-α (data not shown), corroborating that the role of KC and CXCR2 in endothelial recovery is attributable to effects on endothelial migration.

**Discussion**

We found that inhibition of KC by a blocking antibody resulted in enhanced plaque formation after wire-induced carotid injury in apoE−/− mice. This was associated with a reduced rate of endothelial recovery in the KC mAb-treated mice, whereas the relative macrophage and SMC content in the plaques remained unchanged. Macrophages in the plaque were a prominent source of KC during wire-induced plaque formation, albeit endothelial cells covering the plaque area also showed distinctive expression of KC and its receptor CXCR2. In vitro assays of endothelial injury and chemotaxis confirmed that endothelial cell–derived and exogenous KC both promote endothelial wound repair and migration in a process dependent on endothelial CXCR2. Together, these data implicate a crucial role of this chemokine-receptor pair in endothelial recovery after vascular injury.

Monocyte infiltration of the vessel wall is critical for early atherogenesis and plaque growth in advanced stages of atherosclerosis. Firm adhesion of circulating monocytes guided by molecules of the adhesion cascade, such as selectins, adhesion molecules, and chemokines, to the activated endothelium serves as a prerequisite for monocyte infiltration. KC has been determined as the critical and limiting factor for very late antigen-4 mediated monocyte arrest on early atherosclerotic endothelium in isolated perfused murine carotid arteries, confirming in vitro studies which described a similar role for the human ortholog GRO-α in monocyte arrest on tumor necrosis factor-stimulated endothelium. Deletion of KC receptor CXCR2 in bone marrow cells of low-density lipoprotein receptor−/− mice has been demonstrated to reduce lesion size and macrophage infiltration, implying a role of CXCR2 and its ligands in atherosclerotic lesion development. Neointima formation after endothelial denudation is aggravated by monocyte infiltration, therefore a similar role for KC in monocyte recruitment after acute vascular injury compared with native atherosclerosis could be expected. Surprisingly, blockade of KC resulted in a marked increase in plaque area, without significantly altering the cellular plaque composition. This strongly suggests that KC is only marginally involved in monocyte recruitment after endothelial denudation in vivo. Moreover, KC blockade reduced endothelial recovery of the denuded vessel surface, which is inversely correlated to neointimal thickening and SMC proliferation. This finding is compatible with the notion that KC has a protective role in neointima formation by accelerating endothelial recovery. Although KC is also known as an important chemoattractant for neutrophils, its absence from the vessel wall immediately after injury suggests that KC blockade is unlikely to exert its effect by influencing the recruitment of neutrophils or monocytes in the earlier phase of the plaque formation. This further implies that the effect of KC blockade manifests itself only after reendothelialization has started, that is, in the presence of regenerating endothelial cells expressing KC and its receptor CXCR2.

As shown by Evans blue staining, endothelial recovery was almost complete 3 weeks after injury in control mice; however, several small and irregular defects remained detectable in particular in KC mAb–treated mice. This may be because of a local impairment in endothelial recovery or a focal loss of endothelial cell clusters rather than because of a
homogenous delay in the advancement of endothelial fronts. Possible differences in the time to complete recovery compared with the original report by Lindner et al.\(^9\) may be explained by a more severe degree of injury using a coated wire in our model or by slower reendothelialization in apoE\(^-/-\) mice.

The effect of KC on endothelial recovery may outweigh its function on monocyte or neutrophil recruitment, because the reendothelialized vessel surface might be more effective in excluding leukocyte trafficking into the vessel wall.\(^2\) Furthermore, other chemokines, such as monocyte chemoattractant protein-1, which is rapidly upregulated in vascular SMCs after mechanical injury, may guide early monocyte recruitment on surface-adherent platelets.\(^4\) During neointimal growth, monocyte infiltration may alternatively be driven by other chemoattractants, such as RANTES\(^6\) or microimmunofluorescence.\(^12\) Therefore, monocyte recruitment to arteries devoid of the endothelium appears to substantially differ from that in native atherosclerosis with respect to the involvement of KC.

It is well established that CXC chemokines containing the ELR motif are potent angiogenic factors able to stimulate endothelial cell chemotaxis\(^18\) through CXCR2.\(^19\) KC and MIP-2 represent the ligands for CXCR2 in the mouse, and enhanced KC expression by a murine tumor cell line has been associated with a higher density of tumor vessels.\(^20\) A novel and important role of KC and its receptor CXCR2 in endothelial recovery is supported by several lines of evidence: (1) endothelial cell–derived and exogenous KC promote endothelial wound repair after injury; (2) KC can trigger endothelial cell chemotaxis in a Transwell-filter migration assay; (3) the effects of KC were inhibited by blocking CXCR2 but not influenced by ablating VEGF functions. Moreover, endothelial cell proliferation was unaltered by interference with KC or CXCR2. Hence, blockade of KC appears to exert its effects by directly impairing endothelial migration without involving other angiogenic pathways possibly stimulated by CXCR2 activation.

An increase in endothelial progenitor cells after treatment of mice with statins has recently been associated with accelerated reendothelialization and decreased neointima formation after arterial injury.\(^21\) Moreover, intravenous transfusion of in vitro differentiated endothelial progenitor cells has been found to reduce neointima formation in splenectomized mice.\(^22\) Hence, KC blockade may enhance lesion formation and delay reendothelialization by interfering with the recruitment of endothelial progenitor cells from the circulation.

In summary, KC expressed by lesional macrophages and endothelial cells exerts a protective effect on plaque formation after endothelial denudation presumably by accelerated reendothelialization. Therefore, promoting endothelial repair by modulating or enhancing KC responses appears to be a suitable strategy for the prevention of restenosis.

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Figure I: Involvement of KC and CXCR2 in endothelial cell chemotaxis. Chemotaxis of murine endothelial cells (SVEC) in a gelatin-coated transwell filter system. Cells pretreated with CXCR2 mAb or isotype control were allowed to migrate to the bottom side of the filter in response to KC in the presence or absence of KC mAb, VEGF Ab or isotype control in the bottom chamber. Cells were stained with 0.1% crystal violet, extracted with 10% acetic acid and absorbance was read at 620 nm. *P<0.0001 vs. KC alone (n=3 independent experiments).