Molecular Cardiology

Reduced Connexin43 Expression Limits Neointima Formation After Balloon Distension Injury in Hypercholesterolemic Mice

Christos E. Chadjichristos, PhD*; Christian M. Matter, MD*; Isabelle Roth; Esther Sutter; Graziano Pelli; Thomas F. Lüscher, MD; Marc Chanson, PhD; Brenda R. Kwak, PhD

Background—Reducing the expression of the gap junction protein connexin43 (Cx43) inhibits the progression of atherosclerosis, a chronic inflammatory disease. Furthermore, acute vascular injury induced by percutaneous coronary interventions is associated with increased Cx43 expression in neointimal smooth muscle cells (SMCs). However, the relevance of Cx43 after acute vascular injury remains unclear.

Methods and Results—To investigate whether reducing Cx43 expression would affect neointima formation in vivo, we subjected hypercholesterolemic Cx43+/− LDL receptor–deficient (LDLR−/−) mice and Cx43+/+ LDLR−/− control littermates to carotid balloon distension injury, which induced marked endothelial denudation and activation of medial SMCs. We observed decreased macrophage infiltration in Cx43+/− LDLR−/− mice 7 days after injury. Similarly, peritoneal macrophages isolated from Cx43+/− LDLR−/− mice showed reduced migration in vitro compared with Cx43+/+ LDLR−/− macrophages. Interestingly, Cx43+/+ LDLR−/− macrophages also displayed decreased chemotactic activity for SMCs. In addition, we observed less SMC infiltration and proliferation in Cx43+/− LDLR−/− mice 7 and 14 days after balloon angioplasty. Likewise, Cx43+/+ LDLR−/− SMCs showed decreased proliferation and migration in vitro compared with Cx43+/+ LDLR−/− cells. All these events resulted in a reduction of neointimal thickening after vascular injury in Cx43+/+ LDLR−/− mice.

Conclusions—The present study shows for the first time that reducing Cx43 limits neointima formation after acute vascular injury by decreasing the inflammatory response and reducing SMC migration and proliferation. Thus, decreasing Cx43 expression may offer a novel therapeutic strategy for reducing restenosis after percutaneous coronary intervention. (Circulation. 2006;113:2835-2843.)

Key Words: restenosis ■ ion channels ■ connexins ■ inflammation ■ smooth muscle

Percutaneous coronary interventions (PCIs) are commonly used to treat critically narrowed atherosclerotic coronary arteries, yet their long-term efficacy is limited by restenosis or renarrowing of the arteries at the site of the intervention.1 An established way to reduce restenosis has been the deployment of coronary stents; however, despite the frequent use of these devices, including drug-eluting stents, restenosis still represents a serious clinical problem.2

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Restenosis is considered a local vascular manifestation of a general biological response to injury. Initially, both the intervention, by stretching a diseased artery, and the metal of the stent itself induce an exaggerated response to injury that involves recruitment and infiltration of leukocytes to the damaged site, as well as a surge in cytokines and growth factors. Subsequently, vascular smooth muscle cells (SMCs) undergo a phenotypic modulation from a contractile to a synthetic phenotype, proliferate within the media, and migrate toward the intima. These changes are associated with modulation of the extracellular matrix. The sum of all these events leads to neointima formation.3,4 In the past few years, investigators have focused on the paracrine signaling mechanisms that mediate the response to vascular injury, and therapeutic strategies have been developed accordingly.5 However, the clinically proven scope of antirestenotic agents is limited, and additional strategies are needed.
Another form of cell-to-cell interaction involves direct exchange of small cytosolic components via gap junctions, a process referred to as gap junctional intercellular communication. 5-9 Gap junction channels are formed by the multimeric assembly of connexins (Cx), a family of conserved proteins of which >20 members have been reported in mammalian cells. 10 The major gap junction protein expressed in vascular SMCs is connexin 43 (Cx43). Interestingly, the synthetic SMC phenotype is known to express considerably higher levels of this protein compared to contractile SMCs, 11 which suggests that the level of Cx43 expression critically depends on SMC phenotype. Furthermore, regions of intimal thickening showed enhanced Cx43 expression in intimal SMCs of atherosclerotic human and murine arteries. 12,13 These findings indicate that increased Cx43 expression is intimately linked to intimal hyperplasia in the context of chronic injury that promotes atherosclerosis. The correlation between Cx43 and neointima formation in the setting of acute vascular injury has been first investigated by Yeh et al. 14 Balloon injury in the rat carotid artery induced the expression of Cx43 in the media and intima in parallel with SMC activation and phenotype. 15 Because macrophages and other leukocytes account for only a small percentage (≤1%) of the total cell number in the neointima of balloon-injured rat carotid arteries, 15 the authors proposed that Cx43 communication enabled SMCs to coordinate synthesis of extracellular matrix components, to maintain the functional integrity of the media, and/or to regulate repair and neointimal formation in injured vessels. 16 Enhanced Cx43 expression in vascular SMCs and neointimal macrophages was reported later in restenotic lesions of injured rabbits. 17,18 Indeed, in vitro studies confirmed the presence of Cx43 in stimulated primary macrophages or in macrophage cell lines. 19,20 However, the formation of functional gap junction channels in leukocytes is not clearly established. 21 Recently, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) were reported to decrease the enhanced neointimal expression of Cx43 observed after balloon injury in rabbit iliac arteries, which suggests that this protein may play a role in the antiproliferative effect of the compound. 22,23 However, statins are well-known for their pleiotropic antiinflammatory and antiproliferative effects in addition to their actions on Cx43. 24,25 Although there is indirect evidence linking increased neointimal Cx43 expression in macrophages or SMCs to the repair process after vascular injury, the direct involvement of Cx43 in this context is still a matter of debate.

Here, we studied intimal hyperplasia after acute mechanical injury in mice heterozygous for Cx43 (Cx43+/−). To mimic human restenosis that involves both leukocytes and SMCs, we enhanced the inflammatory response to injury using low-density lipoprotein receptor-deficient (LDLR−/−) mice fed a high-cholesterol diet. Using this hypercholesterolemic mouse model of carotid balloon distension injury, we observed restricted neointima formation in Cx43+/−LDLR−/− mice compared with their Cx43+/+LDLR+/+ litters. This phenotype was associated with decreased macrophage infiltration and reduced SMC proliferation at the damaged site. The results indicate that Cx43 is a target for limiting neointima formation after mechanical vascular injury.

### Methods

**Mouse Carotid Balloon Distension Injury**

LDLR−/− mice were crossed with heterozygous Cx43 mice (Cx43+/−) to create a Cx43+/−LDLR−/− mouse line on a C57BL/6 genetic background as described previously. 26 Adult male Cx43+/−LDLR−/− mice, expressing reduced levels of Cx43, and Cx43+/+LDLR+/+ intermated controls (mean weight ~24 g, age 10 weeks) were exposed to an atherogenic diet (1.25% cholesterol, no cholate; Research Diets, New Brunswick, NJ) 7 weeks before balloon distension injury and which was continued until they were euthanized. In addition, the animals received aspirin (16 mg · kg−1 · d−1) starting 7 days before surgery until they were euthanized, to prevent acute vessel closure. Mice were anesthetized with an intraperitoneal injection of ketamine (75 to 95 mg/kg) and xylazine (4 to 8 mg/kg). Balloon injury of the left proximal carotid artery was performed by adapting a previously published protocol, 27 as described in the online Data Supplement.

Mice were euthanized 1 hour, 1 day, 4 days, 7 days, and 14 days after injury. For the latter 2 time points, animals received an intraperitoneal injection of bromodeoxyuridine (BrdU, 50 mg/kg; Sigma, St Louis, Mo) 17 hours, 9 hours, and 1 hour before being euthanized, as described previously. 28 Blood vessels were rinsed in situ with PBS, followed by perfusion fixation with 4% paraformaldehyde (Sigma) at 100 mm Hg for 8 minutes. After postfixation in 4% paraformaldehyde for 2 hours and overnight immersion in 30% sucrose, vessels were embedded in OCT compound (Tissue-Tek, Sakura, Netherlands) and snap-frozen. For immunohistochemical analyses, vessels were rinsed with PBS, embedded in OCT compound, and snap-frozen without fixation. Animal experiments were approved by the local Veterinary Office and performed according to institutional guidelines.

**Morphometry**

Three serial cryosections (5 μm thickness, 300 μm apart) were taken from the middle portion of the dilated segment and analyzed (Carl Zeiss, Oberkochen, Germany) for lumen areas, total intimal cells, and proliferation rates. Cell number was counted from 4′,6-diamidino-2-phenylindole–colored nuclei (DAPI, Boehringer, Mannheim, Germany). Morphometric analysis of areas was performed on these sections after visualization of arterial elastic laminae with Evans Blue (0.3%) staining and fluorescent light. For this purpose, sections were photographed, digitized, and analyzed with Zeiss KS400 software (Feldbach, Switzerland).

**Immunohistochemistry**

Serial cryosections (5 μm) were obtained from injured carotid arteries and immunostained with antibodies recognizing Cx43, α1-smooth muscle actin, and von Willebrand factor as described previously. 25,29,30 Further details are available in the Data Supplement.

**Cell Culture**

Primary vascular SMCs were obtained by enzymatic dissociation of the mouse thoracoabdominal aorta as described previously. 31 Purity of SMCs (>98%) was checked by immunostainings for α1-smooth muscle actin. SMCs were used for proliferation and chemotaxis assays from the second to fourth passages. Peritoneal macrophages were isolated 4 days after intraperitoneal injection of 1 mL of 4% thioglycollate (Sigma) as described previously 32 and used for chemotaxis assays. Purity of peritoneal macrophages (>99%) was secured by magnetic cell sorting with CD11b microbeads and LS columns (Miltenyi Biotec, Gladbach, Germany) and verified by immunostainings for CD68. Conditioned medium was obtained from macrophages seeded at 1.5×10^6 cells per cm², cultured for 48 hours in 1.5 mL of RPMI 1640 medium (Invitrogen Corp, Paisley, Scotland). Cultures were counterchecked for equal numbers of Cx43+/−LDLR−/− and Cx43+/+LDLR+/+ macrophages at this time.

**Migration Assays**

All migration assays were performed as described in the Data Supplement.
Characteristics of Mice Before and After Diet

<table>
<thead>
<tr>
<th></th>
<th>Cx43&lt;sup&gt;−/−&lt;/sup&gt;LDLR&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Cx43&lt;sup&gt;+/−&lt;/sup&gt;/LDLR&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>Cholesterol, mg/dL</td>
<td>Before diet: 310±33</td>
<td>274±25</td>
</tr>
<tr>
<td></td>
<td>After diet: 849±96</td>
<td>837±86</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>Before diet: 251±25</td>
<td>215±20</td>
</tr>
<tr>
<td></td>
<td>After diet: 660±96</td>
<td>630±89</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>Before diet: 24.0±0.4</td>
<td>23.8±0.6</td>
</tr>
<tr>
<td></td>
<td>After diet: 29.6±0.6</td>
<td>29.3±0.7</td>
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Mice consumed a high-cholesterol diet for 7 weeks. Values are mean±SEM. Analyses were performed on 10 mice per group.

**Proliferation Assays**
Primary SMCs were seeded on 96-well plates (5×10<sup>3</sup> cells per well) and processed for proliferation tests with the cell titer 96AQ kit (Promega, Madison, Wis) as described previously<sup>33</sup>.

**Reverse Transcription–Polymerase Chain Reaction**
Total RNA extraction and polymerase chain reaction analysis were performed as described in the Data Supplement.

**Western Blotting**
Western blotting of protein extracted from primary SMCs was performed as described previously<sup>33</sup> with antibodies against Cx43 and anti-mouse β-actin.

**Statistical Analysis**
An unpaired t test was used to compare differences between groups. Probability values <0.05 were considered statistically significant. Values are given as mean±SEM.

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The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

**Neointima Formation After Balloon Injury in Cx43<sup>+/−</sup>/LDLR<sup>−/−</sup> Mice**
To evaluate the role of Cx43 in neointima formation, Cx43<sup>+/−</sup>/LDLR<sup>−/−</sup> and Cx43<sup>+/−</sup>/LDLR<sup>−/+</sup> mice were subjected to carotid balloon distension injury. Similar to previously published studies,<sup>25,26</sup> male Cx43<sup>−/−</sup>/LDLR<sup>−/−</sup> and Cx43<sup>+/−</sup>/LDLR<sup>−/−</sup> mice had comparable body weights and plasma levels of cholesterol and triglycerides at 10 weeks of age on normal chow diet (Table). Vascular inflammation was induced by feeding the mice a cholesterol-rich diet for 7 weeks, after which carotid balloon distension was performed. Fourteen days later, mice were euthanized, and carotid arteries were subjected to morphometric analysis. This time point was chosen for its significant neointima formation and medial thickening in the control Cx43<sup>+/−</sup>/LDLR<sup>−/−</sup> mice (Figure 1A and Figure I). Quantitative analysis revealed a significant increase (<<sup>P</sup> = 0.05) in lumen area in Cx43<sup>−/−</sup>/LDLR<sup>−/−</sup> mice compared between Cx43<sup>−/−</sup>/LDLR<sup>−/−</sup> controls (Figure 1A). Quantitative analysis also revealed a significant increase (<<sup>P</sup> < 0.05) in lumen area in the Cx43<sup>+/−</sup>/LDLR<sup>−/−</sup> mice (Figure 1B), which is indicative of reduced neointima formation. In concordance with this, the counts of total neointimal nuclei were significantly lower (<<sup>P</sup> < 0.05) in Cx43<sup>+/−</sup>/LDLR<sup>−/−</sup> mice than in control animals (Figure 1C).

**Figure 1.** Reduced neointima formation in Cx43<sup>+/−</sup>/LDLR<sup>−/−</sup> mice. A, Cross sections of carotid arteries 14 days after balloon distension injury were stained with DAPI/Evans Blue to outline nuclei and elastic laminas. Interestingly, neointima formation in Cx43<sup>−/−</sup>/LDLR<sup>−/−</sup> mice was markedly reduced compared with Cx43<sup>+/−</sup>/LDLR<sup>−/−</sup> controls (Figure 1A and Figure I). Quantitative analysis revealed a significant increase (<<sup>P</sup> < 0.05) in lumen area in the Cx43<sup>+/−</sup>/LDLR<sup>−/−</sup> mice (Figure 1B), which is indicative of reduced neointima formation. In concordance with this, the counts of total neointimal nuclei were significantly lower (<<sup>P</sup> < 0.05) in Cx43<sup>+/−</sup>/LDLR<sup>−/−</sup> mice than in control animals (Figure 1C). No significant changes in body weight and plasma...
lipid profiles were observed between Cx43−/−LDLR−/− and Cx43+/−/LDLR−/− mice 14 days after balloon injury (Table). These data suggest that targeting Cx43 expression decreased balloon-induced intimal hyperplasia in hypercholesterolemic mice.

**Cx43 Expression After Balloon Injury in Cx43+/−LDLR−/− Mice**

To evaluate Cx43 expression in Cx43+/−LDLR−/− and Cx43+/−/LDLR−/− mice, we performed immunofluorescence on cryosections of the dilated vessels at various time points after balloon distension injury. As expected, injured vessels in Cx43+/−/LDLR−/− mice revealed marked increases in Cx43 expression over time (Figure 2, left). Indeed, Cx43 immunoreactivity was enhanced in the media 4 days after injury. The expression of Cx43 showed a further increase adjacent to the internal elastic lamina of the vessel at 7 days after injury. By 14 days after injury, the neointima had developed as a semicircular mass along the internal elastic lamina (see Figure 1A for an example). Cx43 immunoreactivity was clearly increased in the neointima at this stage. Cx43 immunostaining was also detected in Cx43+/−/LDLR−/− injured vessels, however, its induction after injury was much reduced (Figure 2, right). No labeling was apparent in negative controls (Figure II). These data are consistent with the idea that reduction of Cx43 expression decreased neointima formation.

**SMC Proliferation After Balloon Injury in Cx43+/−LDLR−/− Mice**

To obtain more insight into the cellular events leading to reduced neointima formation, we first focused our investigation on the vascular SMC, a well-known player in the restenosis process. Immunolabeling with α-smooth muscle actin antibodies on carotid cross sections revealed a clear but irregular staining for medial SMCs at 4 and 7 days after balloon injury in both Cx43+/−/LDLR−/− and Cx43+/−/LDLR−/− vessels (Figure 3A, bottom and middle panels). At 14 days after injury, considerably less neointimal SMC staining was observed in the Cx43+/−/LDLR−/− mice than in the Cx43+/−/LDLR−/− mice (Figure 3A, top panels).

To investigate whether reduced neointima formation in Cx43+/−/LDLR−/− mice is caused by reduced SMC proliferation
in the injured carotids, we injected mice with BrdU 17 hours before they were euthanized and determined the proliferation index on serial cross sections 7 and 14 days after injury (Figure 3B and Figure III). The medial proliferation index was significantly lower (P<0.05) in the Cx43+/−LDLR−/− group 7 days after the injury (Figure 3B). At 14 days after balloon injury, significantly reduced BrdU staining was found both in the media and neointima of Cx43+/−LDLR−/− vessels compared with Cx43+/+LDLR−/− vessels. These in vivo data indicate that Cx43+/−LDLR−/− mice exhibit reduced SMC proliferation, which contributes to a reduction in neointima formation in these mice after balloon injury.

Proliferation and Migration of SMCs From Cx43+/−LDLR−/− Mice

To confirm reduced proliferation in the Cx43+/−LDLR−/−-injured vessels, we performed in vitro proliferation experiments using primary SMCs isolated from aortas of both genotypes. Differences in Cx43 expression levels of these primary SMCs were confirmed by Western blot (Figure 4A). As expected, Cx43+/−LDLR−/− SMCs expressed about half the Cx43 compared with Cx43+/+LDLR−/− SMCs (P<0.05; Figure 4B). As shown in Figure 4C, a significant decrease (P<0.05) in proliferation of the Cx43+/−LDLR−/− SMCs was observed compared with Cx43+/+LDLR−/− SMCs. Although these results suggest that reducing Cx43 decreased medial and neointimal SMC proliferation after balloon injury, they do not exclude the possibility that SMC migration might also be affected. To address this possibility, we performed in vitro chemotaxis assays using primary aortic SMCs from both genotypes. These assays revealed a significant and dose-dependent decrease (P<0.01) in migration of SMCs from Cx43+/−LDLR−/− vessels in response to platelet-derived growth factor (PDGF-BB; Figure 4D). Together, these in vivo and in vitro data suggest that Cx43+/−LDLR−/− mice exhibit reduced SMC migration toward the intima, which contributes to a reduction in neointima formation in these mice after balloon injury.

Macrophage Infiltration After Balloon Injury in Cx43+/−LDLR−/− Mice

We next focused our investigations on macrophages, a cell type known to play a crucial role in early events after balloon injury. CD68 immunolabeling on carotid cross sections failed to detect macrophages before 4 days after angioplasty. Macrophages were first observed 7 days after balloon distension in both groups of mice (Figure 5A). At this time, macrophage infiltration was significantly enhanced (P<0.05) in the media of Cx43+/+LDLR−/− mice compared with Cx43+/−LDLR−/− mice (Figure 5B). Macrophage infiltration in the vessels was further augmented with growth of the neointima in both groups of mice (Figure 5A).

To evaluate whether expression of Cx43 in activated macrophages contributed to this phenotype, we performed immunofluorescence for this connexin on peritoneal macrophages isolated from Cx43+/+LDLR−/− and Cx43+/−LDLR−/− mice. As shown in Figure 6A, the presence of Cx43 in the cell membrane of macrophages was confirmed by confocal microscopy. We found a stronger Cx43 fluorescent signal in Cx43+/−LDLR−/− macrophages than in Cx43+/+LDLR−/− cells. To confirm this tendency, the relative amount of mRNA for Cx43 was evaluated by semiquantitative reverse transcription–polymerase chain reaction performed in peritoneal macrophages from both genotypes. As shown in Figure 6B, Cx43+/−LDLR−/− macrophages expressed less Cx43 mRNA than those obtained from Cx43+/+LDLR−/− mice. We then investigated whether reduced Cx43
expression might affect the migration of macrophages in vitro using a modified Boyden chamber (Figure 6C). In response to macrophage chemotactic protein-5, migration of Cx43^-/- LDLR^-/- peritoneal macrophages was markedly reduced (P<0.001) compared with Cx43^+/+LDLR^+/- mice. Together, these in vivo and in vitro data suggest that the reduction in Cx43 expression decreased macrophage infiltration at the injured site.

**Chemotactic Activity of Macrophages From Cx43^+/+LDLR^+/- Mice**

Macrophages are known to secrete chemotactic factors to attract SMCs toward the injured site. To investigate whether macrophages expressing reduced Cx43 levels display altered secretory activity of chemotactic molecules, we prepared conditioned medium from Cx43^-/-LDLR^-/- and Cx43^+/+LDLR^+/- macrophages. This medium was then used as a chemotactant for in vitro chemotaxis assays of primary SMCs. As shown in Figure 7, we observed increased migration of Cx43^-/-LDLR^-/- SMCs in response to Cx43^-/-LDLR^-/- conditioned medium compared with conditioned medium prepared from Cx43^-/-LDLR^-/- macrophages. In addition, the differences in migration of the 2 types of SMCs observed in response to PDGF-BB was retained in response to Cx43^-/-LDLR^-/- conditioned medium but not in response to Cx43^-/-LDLR^-/- conditioned medium. This further illustrates subthreshold levels of chemotactants in the Cx43^-/- LDLR^-/- conditioned medium. These data indicate that macrophages from Cx43^-/-LDLR^-/- mice secrete fewer chemotactant factors, which results in reduced SMC infiltration to the damaged site, which further contributes to a reduction in neointima formation in these mice after balloon injury.

**Reendothelialization in Cx43^+/+LDLR^+/- Mice**

Rapid endothelial repair after PCI is essential to prevent thrombotic complications and inhibit further neointimal growth. To study vascular reendothelialization after balloon injury in mice, we performed immunolabeling with anti–von Willebrand factor antibodies on serial cross sections from the injured carotids. No von Willebrand factor immunolabeling was detected 4 and 7 days after injury in either group of mice. Although von Willebrand factor immunostaining was absent 14 days after injury in all Cx43^-/-LDLR^-/- mice, complete endothelial repair was observed at this time in Cx43^+/+LDLR^+/- mice (Figure 8). These data indicate that the decreased inflammation and reduction in proliferation and migration of vascular SMCs led to accelerated endothelial repair after balloon injury in Cx43^+/+LDLR^+/- mice.

**Discussion**

In the present study, we investigated the role of Cx43 in response to acute vascular injury using genetically modified mice. We found that hypercholesterolemic mice with reduced levels of Cx43 showed restricted neointima formation after balloon distension injury compared with mice with normal Cx43 levels. In addition, we observed that the restraint of intimal hyperplasia was associated with reduced inflammatory and proliferative responses at the site of injury. Restenosis, an exaggerated arterial repair after injury imposed during PCI, has been the principal drawback of this treatment since its introduction more than 25 years ago. The study of structural and cellular mechanisms that lead to this pathology in

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**Figure 5.** Reduced macrophage infiltration in Cx43^-/-LDLR^-/- mice. A, CD68 immunostains (green) were performed on cryosections from balloon-injured carotid arteries of Cx43^-/-LDLR^-/- mice (right) and their Cx43^+/+LDLR^+/- littermate controls (left) killed 4, 7, and 14 days after injury. Elastic laminas were counterstained with Evans Blue (red). Magnification x100 for the inner panels and x650 for the outer panels. Photographs are representative of vessels from 5 mice at each time point in both groups. B, Histogram shows quantification of the CD68-positive macrophage-specific signal in the media of the vessels 7 days after injury. Values are expressed as mean±SEM, n=4. *P<0.05.
humans is hindered by the complexity of the disease and the inability to sequentially characterize restenotic lesions in an individual patient. Therefore, several animal models (pig, rabbit, and rat) have been developed with mechanical, electrical, and chemical injuries to arteries. The potential for genetic manipulations in mice has made this species particularly attractive to identify the key molecules involved in restenosis. In the present study, we fed LDLR-deficient mice a Western diet to induce hypercholesterolemia and subsequently exposed the left common carotid artery to balloon distension injury. Similar to the human situation, this type of injury induced distinct endothelial denudation, followed by leukocyte recruitment and medial SMC activation at the site of intervention.

Our findings are in agreement with previous studies in the rat and rabbit, which reported balloon-induced increases in Cx43 expression in vascular SMCs and macrophages, respectively. Similar to the present results in the mouse, balloon injury in the rat carotid artery was associated first with increased Cx43 expression in the subluminal zone of the media, the major site from which neointimal SMCs are recruited. Thus, Cx43-mediated gap junctional intercellular communication between SMCs may play a role in the initiation of proliferative and migratory responses. Apart from endothelial cells, which are removed during the balloon injury, other possible Cx43-expressing cell types include leukocytes, in particular macrophages. In contrast to the rat carotid model, macrophages are an important constituent of the response to injury in the hypercholesterolemic mouse model used in the present study. Thus, early increases in Cx43 expression in the subluminal zone of the media and the presence of this protein in the neointima of

![Figure 6](image)

**Figure 6.** Reduced migration of Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> macrophages. A. Confocal microscopic images of Cx43 immunostainings (green) in peritoneal macrophages obtained from Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> mice and their Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> littermate controls. Cells were counterstained with Evans Blue (red). Magnification ×630. B. Semiquantitative reverse transcription-polymerase chain reaction for Cx43 and GAPDH expression in purified peritoneal macrophages obtained from Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> mice (+/−) and their Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> littermate controls (+/+). Cx43 cDNA was detected at 430 bp and GAPDH at 200 bp. MW indicates 100-bp molecular weight marker. C. Chemotaxis assays were performed on macrophages obtained from Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> mice (hatched bars) and their Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> littermate controls (solid bars). Recombinant mouse macrophage chemoattractant protein-5 was used as a chemoattractant. Values are expressed as mean±SEM, n=5 (triplicates). ***P<0.001.

![Figure 7](image)

**Figure 7.** Reduced chemoattractive activity of Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> macrophages. Migration assays were performed on SMCs obtained from Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> mice (hatched bars) and their Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> littermate controls (solid bars). Conditioned (cond.) media prepared from cultured isolated peritoneal macrophages of both genotypes were used as chemoattractants. Values are expressed as mean±SEM, n=6 (in triplicate). *P<0.05, **P<0.01.

![Figure 8](image)

**Figure 8.** Reendothelialization in Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> mice. von Willebrand factor immunostains (green) have been performed on cryosections from balloon-injured carotid arteries of Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> mice and their Cx43<sup>+/−</sup>-LDLR<sup>−/−</sup> littermate controls killed 4, 7, and 14 days after injury. Elastic laminas were counterstained with Evans Blue (red). Magnification ×100. Photographs are representative of vessels from 5 mice at each time point in both groups.
balloon-injured mouse carotid arteries may be explained in part by the expression of Cx43 in macrophages in addition to its expression in SMCs.

An important observation in the present study is the reduced macrophage infiltration in the media of the Cx43<sup>+/−</sup>-LDLR<sup>+/−</sup>-carotid arteries 7 days after balloon distension. In addition, the present in vitro data suggested that the reduction in Cx43 expression in peritoneal macrophages correlated with a restricted migratory response, thereby contributing to decreased arterial inflammation in Cx43<sup>+/−</sup>-LDLR<sup>+/−</sup>-mice. Interestingly, neointimal expression of Cx43 has been associated with RAM11-positive macrophages in a rabbit experimental model of restenosis. Interestingly, neointimal expression of Cx43 has been associated with RAM11-positive macrophages in a rabbit experimental model of restenosis. Although in vitro studies revealed intracellular localization of Cx43 in macrophage cell lines, the formation of functional gap junction channels by this protein between these cells is not clearly established. Recently, induction of Cx43 expression in stimulated primary macrophages has been demonstrated in vitro, and the transient formation of functional gap junction channels at inflammatory sites has been proposed. Importantly, we observed Cx43 expression in individual macrophages that cannot form gap junctions. Recent findings suggest that hemichannels open under both physiological and pathological conditions, and this activity may participate in various cellular processes. Consequently, it is possible that Cx43 hemichannels are involved in macrophage recruitment after acute vascular injury.

In addition to the recruitment of macrophages, the proliferation and migration of vascular SMCs are central events in neointimal thickening. Interestingly, Cx43 expression levels in vascular SMCs appear to be intimately linked to their phenotype. Thus, cytokine-induced modulation of vascular SMCs from a contractile to a synthetic phenotype is associated with increased Cx43 expression between the cells. Using primary arterial SMCs obtained from Cx43<sup>+/−</sup>-LDLR<sup>+/−</sup> and Cx43<sup>−/−</sup>-LDLR<sup>−/−</sup> mice, we demonstrate here for the first time a causal relation between Cx43 expression levels and proliferation and migration of these cells. Migration of SMCs implies that these cells have to shed their links with their neighbors and thus loosen their gap junctions. In line with the results of the present study, it has been reported that a decreased rate of neural crest cell migration was associated with loss or reduction of Cx43-mediated gap junctional intercellular communication. In contrast, transfection of Cx43 into the communication-deficient HBL100 cell line increased diapedesis.

These observations suggest an intrinsic difference between primary and tumor cells in the Cx43-related migratory response. Interestingly, this difference between primary and tumor cells also holds true for Cx43-related cell proliferation. Thus, lack of Cx43 promotes tumor growth and cell dedifferentiation. Consequently, retroviral delivery of the Cx43 to human breast tumor cells inhibited tumor growth in vitro. The tumor-suppressing character of Cx43 involves inhibition of the expression of Skp2, a crucial protein that regulates p27 ubiquitination. The decreased proliferation that we observed in response to reduction of Cx43 expression levels in the primary arterial SMCs likely involves other signaling pathways, which remain to be established.

Finally, inflammatory cells that migrate into the damaged site are known to release cytokines and growth factors. We observed that reduced expression of Cx43 in peritoneal macrophages led to altered secretory activity for chemoattractant molecules. Indeed, reduced migration of vascular SMCs was observed in response to Cx43<sup>+/−</sup>-LDLR<sup>+/−</sup> macrophage-conditioned medium compared with that obtained from Cx43<sup>−/−</sup>-LDLR<sup>−/−</sup> macrophages. A similar difference in migration rate of primary SMCs was also observed in response to PDGF-BB, the major growth factor triggering SMC migration. Unfortunately, the concentration of PDGF-BB in the macrophage-conditioned medium was below the detection level (data not shown). Therefore, the chemoattractant factor that is differentially secreted by macrophages of either genotype remains to be identified.

In conclusion, the present study provides for the first time genetic evidence that reducing Cx43 limits neointima formation after acute vascular injury by decreasing the inflammatory response and reducing SMC migration and proliferation. Thus, development of novel strategies that target Cx43 may be of important clinical benefit to confine restenosis after PCI.

Sources of Funding
This work was supported by grants from the Swiss National Science Foundation (No. PPOOA-68883 and No. 3100-06777), the Fondation Prevot, and the Fondation De Reuter to Dr Kwak. Drs Lüscher and Matter are supported by the Swiss National Science Foundation (No. 3100-068118), the European Union (G5RD-CT-2001-00532), and the Bundesamt für Bildung und Wissenschaft (02.0057). Dr Chanson is supported by the Swiss National Science Foundation (No. 3100-068118).

Disclosures
None.

References


**CLINICAL PERSPECTIVE**

The gap junction protein connexin43 (Cx43) plays an important role in atherosclerosis, a chronic inflammatory disease in the vascular wall. Reducing Cx43 expression in mice provides beneficial effects on both the progression and composition of atherosclerotic lesions. Interestingly, acute vascular injury induced by percutaneous coronary interventions is associated with increased Cx43 expression in the neointima; however, the relevance of this protein after acute vascular injury remains unclear. In the present study, we provide genetic evidence for the implication of Cx43 in neointimal hyperplasia after carotid balloon distension injury in mice. Indeed, a reduction in Cx43 expression restricted neointima formation after acute vascular injury by limiting the inflammatory response and the proliferation and migration of smooth muscle cells toward the damaged site. Together, these events resulted in accelerated endothelial repair. Thus, Cx43-mediated intercellular communication may also play a crucial role in confining lesion formation after acute vascular injury. The clinical relevance of this study is 2-fold. First, our findings suggest that Cx43 might be an additional target for local delivery strategies aimed at reducing restenosis after percutaneous coronary interventions. In this respect, recent applications of connexin-specific blocking peptides on smooth muscle cells and isolated blood vessels are of particular interest. Second, wound healing in multiple tissues is associated with altered gap junctional communication or connexin expression. Thus, strategies that target the gap junction protein might be of interest for other diseases as well.
Reduced Connexin43 Expression Limits Neointima Formation After Balloon Distension Injury in Hypercholesterolemic Mice
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Circulation. 2006;113:2835-2843; originally published online June 12, 2006;
doi: 10.1161/CIRCULATIONAHA.106.627703

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/113/24/2835

Data Supplement (unedited) at:
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