Smooth Muscle–Targeted Knockout of Connexin43 Enhances Neointimal Formation in Response to Vascular Injury

Yongbo Liao, Christopher P. Regan, Ichiro Manabe, Gary K. Owens, Kathy H. Day, Dave N. Damon, Brian R. Duling

Objective—Vascular disease alters and reduces connexin expression and a reduction in connexin 43 (Cx43) expression diminishes the extent of atherosclerosis observed in a high-cholesterol diet murine model. We hypothesized that connexins might play a role in the smooth muscle cell response to vascular injury.

Methods and Results—We therefore studied a line of smooth muscle cell-specific, Cx43 gene knockout mice (SM Cx43 KO) in which the carotid arteries were injured, either by vascular occlusion or by a wire injury. In the SM Cx43 KO mice both types of injury manifested accelerated growth of the neointima and of the adventitia. Isolated vascular smooth muscle cells from the SM Cx43 KO mice grew at a slightly faster rate in culture, and to marginally higher saturation densities than those of control mice, but these changes were not adequate to explain the large changes in the injured vessels.

Conclusions—These observations provide direct evidence that smooth muscle Cx43 gap junctions play a multi-faceted role in modulating the in vivo growth response of vascular smooth muscle cells to vascular injury. (Arterioscler Thromb Vasc Biol. 2007;27:1037-1042.)

Key Words: adventitia • atherosclerosis • smooth muscle • thrombus

Interellular communication mediated by gap junctions plays a pivotal role in the cardiovascular system, being involved in such diverse processes as determination of vasomotor tone, cell differentiation, growth control, embryonic development, and coordination of contraction of cardiac muscle cells.1–4 Gap junctions are formed from combinations of 1 or more of the 4 connexin protein isomers that are known to be expressed in the vasculature: Cx37, 40, 43, and 45,5–10 and there is accumulating evidence indicating that the connexins may play a role in a variety of vascular pathologies including: hypertension,11–14 ischemia/reperfusion injury,15 and atherosclerosis.16–22 Moreover, gap junctional communication is reduced in proliferating vascular smooth muscle (VSM) cells,23 suggesting that the connexins might play a role in the modulation of the vascular response to injury or experimental atherogenesis.24

Our studies tested the hypothesis that selective deletion of a particular connexin in VSM would modify the response to vascular injury. To accomplish this, we generated a knockout mouse in which deletion of the Cx43 gene was confined to smooth muscle (SM) cells.25 Here we report the effects of this deletion on the response of the carotid artery to injury, and on the modification of the growth pattern of cultured VSM cells isolated from the SM Cx43 knockout (KO) mice.

Materials and Methods

Generation of Mice With SM Cx43 KO
A line of mice in which the second exon of the Cx43 gene was flanked by loxP sites was produced26 and crossed with a second line of transgenic mice that carried a transgene composed of the SM myosin heavy chain promoter/enhancer and the Cre recombinase gene to induce smooth muscle-restricted Cre expression.25 Crossing these 2 lines generated mice in which the Cx43 gene was deleted from those cells in which myosin heavy chain drove the Cre expression, ie, in SM cells, and resulted in cell specific deletion (supplemental Figure II, available at http://atvb.ahajournals.org). Mice homozygous for the floxed Cx43 gene were used as controls for these experiments (supplemental Figure I). Breeding, housing, maintenance, and experimental procedures were all conducted in accordance with the approved practices of the University of Virginia Animal Care and Use Committee.

DNA Preparation and Analysis
The genotype of each animal was confirmed by polymerase chain reaction analysis using primer sequences described online (see http://atvb.ahajournals.org).

Original received July 7, 2006; final version accepted February 14, 2007.
From the Department of Anesthesiology (Y.L.), Department of Molecular Physiology and Biological Physics (G.K.O., D.N.D., B.R.D.), and Cardiovascular Research Center (K.H.D.), University of Virginia, Charlottesville, Va; Department of Pharmacology (C.P.R.), Merck Research Laboratories, West Point, Pa; Department of Cardiology (I.M.), University of Tokyo, Tokyo, Japan.
Correspondence to Dr Brian R. Duling, Department of Molecular Physiology and Biological Physics, University of Virginia, School of Medicine, MR-4 Building, Room 6051, Charlottesville, VA 22908. E-mail brd@virginia.edu
© 2007 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org

DOI: 10.1161/ATVBAHA.106.137182

1037
Measurement of Blood Pressure and Heart Rate

Tail cuff measurements of systolic blood pressure and heart rate were obtained using a Visitech Systems tail cuff instrument. As shown in supplemental Table I, there was no significant difference in blood pressure of the controls compared with the SM CX43 KO animals.

Occlusion Injury of the Carotid Artery

A common carotid artery injury was produced by ligation as described by Kumar et al. Although the vessel responses to occlusion were altered in the carotids of the SM CX43 KO mice (compare supplemental Figures IIIB and IIIF with IIID and IIIH), the changes were less predictable than those seen in the wire injury model; therefore, we selected the latter treatment for the detailed quantitative analysis.

Denudation of the Carotid Artery Using a Guide Wire

A second type of carotid artery injury was made as described by Lindner. After anesthesia, a transverse arteriotomy was made in the left external carotid artery, and a 0.014-inch flexible angioplasty guide wire (Advanced Cardiovascular Systems, Inc, Temecula, Calif) was introduced and advanced ~1 cm toward the aortic arch. The intima of the left common carotid artery was injured by rotating the wire 3 times during withdrawal and the left external carotid artery was then tied off. The right external carotid artery was ligated as a control. Mice were allowed to recover and returned to the animal care facility.

Tissue Harvest and Morphological Examination

Carotids were obtained from anesthetized mice, fixed, and morphometrics performed to determine: lumen radius, thickness of media, neointimal area, luminal area, medial area, and adventitial area (supplemental Table I).

Immunohistochemistry

Paraffin sections were examined on an Olympus Fluoview, dual-laser, confocal microscope. Specificity of antibodies was confirmed by preincubation of each antibody with the appropriate peptide. Rabbit anti-mouse Cx43 antibody was purchased from Alpha Diagnostic International (Cx43B12-A; San Antonio, Tex). Rabbit anti-human von Willebrand factor antibody and mouse monoclonal SM anti-α actin antibody were obtained from Sigma (St. Louis, Mo). Rat monoclonal anti-CD45 antibody was purchased from BD Biosciences (San Diego, Calif). Further details of immunohistochemistry are described in the online Methods section.

Growth Curve of Cultured VSM Cells

VSM cells were isolated from 3 to 4 aortas (4 to 5 weeks old, male or female) pooled and placed into culture following the protocol of Rovner et al. Only passages 3 to 5 were used for the study. The VSM cells were characterized by immunostaining for SM myosin heavy chain and SM-α actin (Sigma). At daily intervals, plates were trypsinized to release the cells, which were then counted in triplicate on a hemocytometer.

Statistics

All values are expressed as mean±SEM. One-way ANOVA was used for statistical analysis of group comparisons. P<0.05 was considered significant.

Results

Production and Characterization of the SM-Specific Cx43 KO Mice

Mice homozygous for both the floxed Cx43 allele and the myosin heavy chain–Cre gene grew normally and were fertile. Evidence demonstrating the specificity and efficacy of the deletion produced in these mice is presented (supplemental Figures I and II). Cx43 immunostaining was punctuate in the media of arteries from control animals and was greatly reduced in the VSM of SM Cx43 KO mice (Figures 1 and 2, and supplemental Figures IIA to IIE and IIIE, IIIF). These observations are consistent with the demonstration by Regan et al that Cre expression in the myosin heavy chain–Cre transgenic animals is uniform, and is restricted to the SM cells and thus guides cell-specific deletion.

Unexpectedly, the intensity of Cx43 immunostaining in the endothelium of the aorta from the SM Cx43 KO mice was also often reduced or even eliminated in the SM Cx43 KO animals (compare supplemental Figure IIB and IIE). Endo-

Figure 1. Comparison of injury responses of control (A to G) and SM Cx43 KO (B to H) mice to wire injury of the left carotid artery. A and B, Sham-injured, right carotid arteries. C to H, Wire-injured left carotid arteries. A to F, Hematoxylin and eosin stain to visualize the neointima formation and the adventitial proliferation at different magnifications. G and H, Confocal fluorescent images used for the measurement of internal elastic lamina and external elastic lamina. White arrowheads show the locations of the internal elastic lamina. A to D, bar=200; E to H, bar=100.
The albumin was still present in the vessels of SM KO mice as evidenced by the presence of platelet endothelial cell adhesion molecule (PECAM) (supplemental Figure IIB, IIE) and von Willebrand factor labeling (Figure 2B).

**Blood Pressure and Heart Rate**
The SM Cx43 KO mice showed no alteration in blood pressure or heart rate (supplemental Table I).

**Enhanced Proliferation of VSM After Injury in the SM Cx43 KO Mice**
Examples of observations made on the carotids of control and SM Cx43 KO mice before and after wire injury are shown in Figure 1. After sham operation, there was no significant difference between the control and SM Cx43 KO mice (compare Figure 1A and 1B). At 7 days after surgery, no neointima formation was observed in the wire-injured carotid artery of control mice, although there was modest growth in adventitia (compare Figure 1B and 1D). Quantitative measurements confirmed the impression given in Figure 1, with morphological measurements showing significant increases in areas of the neointima, media, and adventitia when the wire-injured carotid arteries of control and SM Cx43 KO mice were compared (Table). Immunostains to determine cell types associated with the changes in vessel wall morphology shown in Figure 1 are presented in Figure 2. Cells in the neointima are, for the most part, SM-like (Figure 2C), although there are occasional endothelial cells (Figure 2B). Leukocytes are occasionally present in the adventitia but not evident in the neointima. Occasional Cx43-positive cells can be seen in the adventitia (Figure 2A).

**Growth Rates of VSM From Control and SM Cx43 KO Mice**
We compared the growth characteristics of control and Cx43 KO VSM cells in culture to evaluate their intrinsic growth rates. Cells isolated from mouse aorta were confirmed to be VSM by positive staining for SM myosin heavy chain and SMα/Hα-actin (supplemental Figure IVA, IVB). Cultured VSM cells from the SM Cx43 KO mice exhibited a VSM phenotype similar to the control cultured cells, and grew logarithmically, although at a slightly faster rate and to a somewhat higher confluent density than those harvested from the control mice (online data, supplemental Figure IVC). Although the deletion of Cx43 gene suggested a slightly elevated proliferation of the VSM, the change in vitro was not nearly as great as that observed in vivo.

**Discussion**
Our findings support and extend the previous evidence that connexin expression plays an important role in regulation of vascular growth in response to injury.16,18,20,24,30–33 Neointimal formation after injury was markedly increased in SM Cx43 KO mice as compared with controls, and VSM cells derived from the KO mice showed slightly enhanced proliferation and density in vitro. These results are consistent with previous studies implicating a role for gap junctional communication.

**Morphological Analysis of Carotid Arteries After Wire Injury**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Floxed Cx43 Mice (n=8)</th>
<th>SM Cx43 KO Mice (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neointimal area</td>
<td>0</td>
<td>8.5±3.2*</td>
</tr>
<tr>
<td>Lumen area</td>
<td>96.2±5.3</td>
<td>114.2±11.7</td>
</tr>
<tr>
<td>Medial area</td>
<td>19.3±2.1</td>
<td>29.0±3.5*</td>
</tr>
<tr>
<td>Adventitial area</td>
<td>17.9±7.0</td>
<td>109.8±19.5†</td>
</tr>
<tr>
<td>Lumen radius</td>
<td>174.6±4.7</td>
<td>188.8±9.5</td>
</tr>
<tr>
<td>Thickness of media</td>
<td>16.8±1.8</td>
<td>22.3±2.7</td>
</tr>
</tbody>
</table>

*Perimeters of lumen, internal elastic lamina, and external elastic lamina were measured from confocal images. Radius and thickness are measured in μm. Area is measured in μm². Data are expressed as mean±SE.

*P<0.05; †P<0.01.
munication in regulation of cell growth, presumably through cell–cell-mediated transfer of signaling molecules or electrical signaling. Our findings suggest that the absence of intact Cx43 signaling disrupts critical feedback control pathways necessary for vascular morphogenesis, as has been shown for the endothelium.34

The success of using the Cre/loxP system in experiments such as these is dependent on 2 factors: (1) that the insertion of loxP does not interfere with the native gene expression; and (2) that the promoter driving Cre expression is cell-specific. Polymerase chain reaction analysis revealed no deletion of the Cx43 allele in the brain gray matter or white blood cells, samples that should contain no SM cells (supplemental Figure I). Most importantly, we examined the efficiency and selectivity of Cx43 deletion in VSM layers by immunohistochemistry. As shown in Figure 2 and supplemental Figure II, the Cx43 immunostain in VSM of aorta and carotid artery was markedly reduced in the media of the SM Cx43 KO mice compared with controls.

Cx43 does not appear to be completely eliminated in the media of the KO mice, presumably reflecting incomplete gene deletion with the cre system. It is noteworthy that cre-based deletion need not be complete if some of the cells fail to express cre at a critical time in development. This is a fact that has received very little experimental scrutiny by those who use the conditional deletion approach and we hope to analyze cre expression in more detail in the future.

Cx43 Gap Junctions Are Critical for the Remodeling Process in Response to Vascular Injury

Restenosis is a combination of neointimal formation and arterial remodeling involving alterations in many processes, including complex interactions among endothelium, SM cells, fibroblasts, and inflammatory cells. Thus, our observations of a striking difference in the intimal growth response between the wild-type and the SM Cx43 KO animals in response to wire injury (Figure 1, Table) are both novel and of great potential significance. The data suggest that disruption of normal gap junctional communication might contribute to other disease states associated with abnormal VSM growth, including hypertension, atherosclerosis, and postangioplasty restenosis, and that the gap junctions might serve as targets for new therapeutic interventions for these human diseases.27,35–37

Our findings are in sharp contrast to the work of Chadjichristos et al.,38 who showed that heterozygous Cx43 KO mice manifested reduced neointimal formation rather than enhanced neointimal formation. Their experimental model included a high-fat diet, thus differences may simply reflect different vascular adaptive processes. In addition, the mice used in the studies by Chadjichristos were global KO mice, thus Cx43 was reduced in all cell types expressing Cx43, and as a result we do not know which cell type initiated and which contributed to the altered atherosclerotic response. The differences between the results in the 2 experiments may reflect complex interactions between different cell types in the global KO.

An incidental but striking finding was that the adventitial area of injured carotid arteries in the SM Cx43 KO mice was 6-times greater than that of control mice (Table, Figure 1F), despite the evidence that the activity of the SMC promoter is restricted to the VSM.25 (supplemental Figure I), suggesting that the effects of Cx43 deletion on adventitial growth were secondary to some change in the SM. Deletion of Cx43 from the VSM might alter the release of paracrine growth factors such as platelet-derived growth factor (PDGF) BB or basic fibroblast growth factor (bFGF), which could be mitogenic for adventitial fibroblasts as well as VSM. Studies by others have shown adventitial reactions in atherosclerosis, arteritis, and after angioplasty.39–40 Moreover, Booth et al.41 reported that manipulation of the vessel wall resulted in lesions that mimic the biochemical and morphological changes observed in early stages of human atherosclerosis. It is thus possible that disruption of Cx43 signaling in VSM in some manner exacerbates vascular inflammation in response to injury and thereby secondarily alters adventitial cell growth.

There are controversial reports suggesting that after injury there is migration of adventitial fibroblasts toward the media and that these cells subsequently contribute to the neointima formation.42,43 In addition, it has been shown that there are pluripotent cells in the adventitia, which may contribute to the growth of the media.44 Perhaps the myofibroblasts within the adventitia were actually derived from the migration of medial SM cells into the adventitia, where they may have undergone phenotypic switching to a fibroblast-like cell.45 However, it has generally been assumed that there is migration into the intima, not into the adventitia. The positive immunostaining with α-actin and CD45, and negative staining with Cx43 antibodies (Figure 2) are consistent with the possibility of migration of medial cells into the adventitia.

Identification of Cell Types in the Neointima

We used multiple antibodies to identify the cell types in the neointima and found that the majority of neointimal cells stained positive for α-actin, although some cells at the luminal surface stained positive with the endothelial cell-specific marker von Willebrand factor. Few CD45-positive cells were seen in the neointima, although CD45-positive cells were diffusely distributed in the adventitia. The majority of the intimal cells express α-SM actin and were thus likely VSM cells (Figure 2B). Negative Cx43 immunostaining of SM cells in the neointima area is consistent with the idea that the augmented neointimal formation in SM Cx43 KO mice was primarily caused by migration of medial SM cells that had undergone Cx43 gene deletion through the Cre/loxP interaction, and not the consequence of secondary alterations in vascular inflammation. These findings are reminiscent of the findings of Kwak et al.31 in atherosclerotic mice.

Interplay of Cx43 Expression Between VSM and Endothelium

Although our data indicate that the Cre-mediated deletion was confined to a single gene in a single cell type, secondary interactions appeared to cause a reduction in the Cx43 protein in the aortic endothelium (compare supplemental Figure IIB and IIE). The PECAM and von Willebrand factor antibody staining indicated that the endothelial cells were still present in the KO mice (Figure 2 and supplemental Figure II). In a
previous report we noted that mice with a cell-specific deletion of the endothelial cell Cx43 showed a reduction of Cx43 message in the adjacent VSM layers. Such a process previously reported in the liver by Nelles et al. and the parallel changes in the Cx43 expression in endothelium of the SM CX43 KO mice emphasize that caution should be used in the interpretation of the specificity of conditional KO experiments, especially when dealing with a protein that is part of a complex.

Coregulation of the Cx43 proteins in the 2 cell types might reflect paracrine signaling or, alternatively, such coregulation might arise as a result of transcellular signaling mediated by myoendothelial junctions. Simon observed such coregulation of Cx40 and 43 in Cx40 KO mice, and thus his findings support a linkage in the mixture of gap junctional proteins. As yet, there has been no determination of the sensitivity of the VSM to injury in vivo. The enhancement of in vitro growth properties of cultured aortic VSM derived from SM CX43 KO mice provides additional evidence that Cx43 plays a role in growth regulation of VSM, but the much smaller in vitro effect argues that additional factors are involved in the determination of the sensitivity of the VSM to injury in vivo. Moreover, the alterations in both intima and adventitia suggest that overall vascular remodeling and morphogenesis are dependent on Cx43 signaling through as yet poorly understood processes.

Sources of Funding
This work was supported by NIH grants HL12792, HL23531, and HL53318 (B.R.D.); grants R01 HL57353 and P01 HI19242 (G.K.O.), the Academic Enhancement program on Gene Transfer and Gene Therapy, and the Robert M. Berne Cardiovascular Research Center at the University of Virginia.

Disclosure
None.

References


Smooth Muscle–Targeted Knockout of Connexin43 Enhances Neointimal Formation in Response to Vascular Injury
Yongbo Liao, Christopher P. Regan, Ichiro Manabe, Gary K. Owens, Kathy H. Day, Dave N. Damon and Brian R. Duling

Arterioscler Thromb Vasc Biol. 2007;27:1037-1042; originally published online March 1, 2007; doi: 10.1161/ATVBAHA.106.137182
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 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/27/5/1037

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2007/03/06/ATVBAHA.106.137182.DC1

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/