Altered Pattern of Vascular Connexin Expression in Atherosclerotic Plaques

Brenda R. Kwak, Flore Mulhaupt, Niels Veillard, Daniel B. Gros, François Mach

Abstract—Paracrine cell-to-cell interactions are crucial events during atherogenesis. However, little is known about the role of direct intercellular communication via gap junctions during this process. We have investigated the expression pattern of 3 vascular gap junction proteins (connexins) in mouse and human atherosclerotic plaques. Low density lipoprotein receptor–deficient mice were fed a high-fat diet for 0, 6, 10, or 14 weeks to induce different stages of atherosclerosis. Connexin37 (Cx37) and Cx40 were detected in the endothelium, and Cx43 was detected in the media of nondiseased aortas. In early atheromas, endothelial and medial connexin expression remained unchanged, and “islets” of Cx43 in smooth muscle cells and Cx37 in macrophages were observed in the neointima. In advanced atheromas, Cx37 was detected in medial smooth muscle cells and in macrophages in the lipid core but not in the endothelium covering the plaques. Cx40 could also no longer be detected in the endothelium covering the plaques. Cx43, on the other hand, was detected in the endothelium covering the shoulder of the plaques and also sparsely in neointimal smooth muscle cells. Similar results were obtained for human carotid arteries. In conclusion, vascular connexins are differentially expressed by atheroma-associated cells within lesions. These observations suggest a role for gap junctional intercellular communication during atherogenesis. (Arterioscler Thromb Vasc Biol. 2002;22:225-230.)

Key Words: atherosclerosis ■ gap junctions ■ connexins ■ endothelium ■ macrophages ■ smooth muscle

Atherosclerosis, the principle cause of death in Western societies, is a progressive disease that generally begins in childhood and has clinical manifestations in middle age or later.1 Atherosclerotic plaques have 3 principal components: (1) cells, including endothelial cells, smooth muscle cells, macrophages, and T lymphocytes; (2) connective tissue extracellular matrix; and (3) intracellular and extracellular lipid deposits. These 3 components occur in varying proportions in different plaques, giving rise to a spectrum of lesions. Typically, the superficial fibrous cap is composed of smooth muscle cells with dense connective tissue and a few inflammatory cells. The cellular area beneath and to the side of the cap, the shoulder, consists of a mixture of macrophages, smooth muscle cells, and T lymphocytes, whereas the deeper necrotic core is a disorganized mass of lipids, cellular debris, and lipid-laden foam cells.2,3

Initiation and progression of the atherosclerotic plaque involve complex patterns of interaction between the cells of the arterial wall, in which cytokines, chemokines, and growth factors are known to play a critical role.4,5 Apart from these paracrine signaling mechanisms, another form of cell-to-cell interaction involves direct intercellular communication via gap junctions. Gap junctions are clusters of transmembrane channels that link the cytoplasmic compartments of neighboring cells, allowing a direct exchange of ions and second messengers.6 A gap junction channel is constructed from a pair of hemichannels named connexons; each connexon is assembled from 6 connexin molecules. The connexins form a multigene family consisting of at least 15 members in mammals.7 Each connexin forms channels with different properties of gating, permeability, and selective interaction with other connexins.8 Within 1 organ or cell type, various connexins can be expressed. Three connexins, connexin37 (Cx37), Cx40, and Cx43, are reported to be expressed in the vascular wall.9–17 Endothelial cells and smooth muscle cells have distinctive but overlapping connexin expression patterns. Whereas Cx37 and Cx40 (and also, depending on the type of vessel and its position in the vascular tree, Cx43) are coexpressed in the endothelium,18,19 smooth muscle cells in the media predominantly express Cx43.20 Some reports suggest that Cx40 may also be expressed in vascular smooth muscle cell types.14,21,22

Current knowledge about vascular gap junctions is growing rapidly. These communication pathways have been implicated in a variety of vascular functions, such as coordination of vasomotor responses, regulation of angiogenesis, repair of the endothelial lining, and senescence.23–27 In addition, connexin expression is altered in vascular disease states such as hypertension and after balloon catheter injury.28–30 However, information regarding the involvement of vascular connexins in atherogenesis is fragmentary. In the early stages of human coronary atherosclerosis, an upregula-
tion of Cx43 in the intimal smooth muscle layer, which was reduced in advanced atheromatous lesions, has been described. Hypercholesteremia-induced atherosclerosis in rabbits resulted in Cx43 expression associated with macrophage foam cells, but there were reduced levels of this connexin between smooth muscle cells. At last, a genetic polymorphism has been recently discovered in the human Cx37 protein that appears to be a prognostic marker for atherosclerotic plaque development. Considering the crucial role of paracrine cell-to-cell interactions in the pathogenesis of atherosclerosis as well as the important roles of gap junction-mediated cell-to-cell communication in vascular physiology, we sought to investigate the expression patterns of the 3 vascular connexins in normal vessels and in advanced atherosclerotic lesions.

Methods

Materials
LDL receptor (LDLR)-deficient (LDLR−/−) mice, aged 10 weeks, with a C57BL/6J background (Jackson Laboratory, Bar Harbor, Me) consumed a high-cholesterol diet (product No. D12108 [1.25% cholesterol and 0% cholate], Research Diets, Inc) for 6, 10, or 14 weeks. Then the mice were euthanized, and their aortas were taken. Age-matched LDLR+/+ mice that were not placed on the diet were used as controls. The aortic arches were perfused with 0.9% NaCl and snap-frozen in OCT compound (Tissue-Tek). Surgical specimens of human carotid atheroma were obtained by

Antibodies
Polyclonal antibodies raised in rabbits against oligopeptides of the carboxy terminus of the gap junction proteins Cx37 (amino acids 315 to 331)19–24 or Cx40 (amino acids 335 to 356)25 were used. Rabbit polyclonal antibodies against Cx43, smooth muscle (SM) myosin heavy chain (MHC), or von Willebrand factor were purchased from Zymed Laboratories, Biomedical Technologies Inc, and Dako, respectively. Mouse monoclonal antibodies against human α-SM actin (SMA), human CD31 (anti–endothelial cell), or HAM-56 (anti-macrophage) and a rat monoclonal antibody against mouse macrophages (MOMA-2) were purchased from Dako, Sigma Immunochemicall, Dako, and Biosource International, respectively.

Immunohistochemistry
For cell identification, serial sections of 5 μm were cut, air-dried, and fixed in acetone for 5 minutes at −20°C. Sections were preincubated with 8% normal horse or goat serum (Sigma) for 15 minutes, then incubated with cell-selective primary antibody (anti-SMA 1:100, anti–von Willebrand factor 1:1000, anti-CD31 1:50, anti–HAM-56 1:100, anti–SM-MHC 1:10, or anti–MOMA-2 1:10) for 90 minutes, followed by biotinylated secondary antibody (anti-rabbit or anti-mouse IgG 1:1000, anti-rat IgG 1:8000, Vector Laboratories) for 45 minutes and avidin–biotin–alkaline phosphatase complex (Vectastain ABC-AP kit), and visualized with Vector red (Vector Laboratories). Sections were counterstained with Harris hematoxylin solution (Sigma) for 8 seconds or with Mayer’s hematoxylin solution (Merck) for 1 minute. All steps were performed at room temperature, and between the incubation steps, the cells were rinsed with PBS. Specificity of the Cx43 labeling was confirmed by preabsorption for 15 minutes at room temperature with its immunogenic peptide (20 μg/mL, Zymed Laboratories). Specificity of the Cx37 or Cx40 labeling was checked by replacing the primary antibody with preimmune serum.

For double fluorescence staining, the protocol described for connexin staining was followed. CD31, SMA, or HAM-56 was detected with an anti-mouse secondary antibody conjugated to FITC (1:1000, Jackson Laboratories) for 4 hours or with biotinylated secondary antibodies (1:100, Vector Laboratories) followed by the detection described above. All steps were performed at room temperature, and between the incubation steps, the cells were rinsed with PBS. Some sections were counterstained with Evans blue solution (0.03% in PBS) for 1 minute. Specificity of the Cx43 staining was followed. CD31, SMA, or HAM-56 was detected with an anti-mouse secondary antibody conjugated to Texas red (1:200, Jackson Laboratories).

Sections were examined on a Zeiss Axiophot microscope equipped with fluorescence and appropriate filters. Photographs were taken on a computer with use of a CCD camera (Photonic Science) and Image Access software (version 2.04). To confirm the coexpression of connexins with cell markers, slides were further examined with a confocal laser scan fluorescence inverted microscope (CLSM 410, Zeiss). Images were processed with Adobe Photoshop 5.0 (Adobe System).

Results
To study the expression pattern of vascular connexins during atherogenesis, we used LDLR−/− mice and exposed them to a high-cholesterol diet for 6, 10, or 14 weeks to induce, in a reproducible manner, atherosclerotic plaques at different stages of development. These atherosclerotic lesions typically developed at lesion-prone sites, such as the lesser curvature of the aortic arch and the outflow tracts of the innominate, left common carotid, and left subclavian arteries. Age-matched LDLR−/− mice without dietary intervention were used as controls and did indeed show no signs of plaque formation at those localizations (Figure 1). Despite some small variations in the histological morphology, lesions after 6, 10, and 14 weeks of dietary intervention could be divided in 3 categories, ie, intimal thickening, early atheroma, and advanced atheroma, respectively. Whereas we observed lipid deposi-
Figure 2. Connexin distribution in nondiseased mouse aorta and at different stages of mouse atherosclerotic plaques: immunofluorescence detection. Photographs of frozen sections (5 μm) of LDLR−/− mice without dietary intervention (A through C) or after a high-cholesterol diet for 6 weeks (D through F), 10 weeks (G through I), or 14 weeks (J through L) are shown. Panels A, D, G, and J show incubation with antibodies against Cx37; panels B, E, H, and K show incubation with antibodies against Cx40; and panels C, F, I, and L show incubation with antibodies against Cx43. Aspecific staining of elastic laminas clearly distinct from punctate-specific connexin staining can be observed in panels B, D, E, and L. Bar—70 μm. EC indicates endothelium; L, lumen of the artery; M, media; and NI, neointima. Similar results were obtained in independent experiments with aortas from 3 to 5 mice in each group.

tion in the vascular wall of all mice after 6 weeks of diet (data not shown), increased intimal thickness was present in only a small fraction of them. Lesions after 10 weeks of diet, defined as early atheroma, include initial fatty streaks as well as intermediate lesions of atherosclerosis. The complex advanced lesions after 14 weeks of dietary intervention shared a number of common features: a superficial fibrous cap of mainly smooth muscle cells, an area containing mainly inflammatory cells in the shoulder of the plaque beneath the cap, and a lipid-rich core.

The expression patterns of Cx37, Cx40, and Cx43 in the different stages of atherosclerosis were investigated in serial sections by immunolabeling with fluorescence detection (FITC, green signal; Figure 2) as well as enzymatic detection (alkaline phosphatase, red signal; Figure 3). In control aortas, a clear Cx37 and Cx40 immunolabeling was observed in the endothelium. No signal for either connxin could be detected in the media at this time point (Figure 2A and 2B). However, Cx43 was abundantly expressed in the medial smooth muscle layer but could not be detected in the endothelium of control aortas (Figure 2C). After 6 weeks of diet, a similar expression pattern was observed for all 3 connexins (Figure 2D-F). After 10 weeks of diet, Cx37 and Cx40 were still present in the endothelial layer (Figure 2G and 2H). In addition, a Cx37 signal was observed in small “islets” in the neointima of these early atheromas (arrowhead in Figure 2G). At this time point, Cx43 expression was observed in the medial smooth muscle layer (Figure 2I) and also appeared in the neointima as small islets at sites different from the Cx37 islets previously mentioned (arrowheads in Figure 2I). In the shoulder area of advanced lesions, endothelial expression of Cx37 and Cx40 was no longer detected (Figure 2J and 2K). In contrast, a punctate labeling of Cx43 was observed in the endothelium in this shoulder region of advanced plaques (arrow in Figure 2L). None of the 3 vascular connexins was detected in the endothelium covering the center of advanced lesions (Table).

To define the cell types expressing the different connexins within atherosclerotic plaques, we performed immunostaining with enzymatic detection on consecutive cryosections. The advantage of enzymatic detection is that histological details of the plaque are preserved. However, this method is less sensitive than fluorescence, leading to an apparent loss of connexin staining in the endothelium. Cx43 immunostaining was colocalized with smooth muscle cells in the media of mouse control aortas (Figure 3A and 3B). As expected, no signal was obtained with the macrophage marker MOMA-2 or with Cx37 antibodies in these nondiseased aortas (Figure 3C and 3D). In early atheromas induced by 10 weeks of a cholesterol-rich diet, Cx43 was observed in smooth muscle cells of the media beneath the plaque (Figure 3E and 3F). In the neointima of early atheromas, Cx43 was colocalized with the smooth muscle cell marker (eg, see arrows in Figure 3E and 3F), whereas Cx37 was colocalized with the macrophage marker (arrows in Figure 3G and 3H). Note that the latter colocalization does not imply that all macrophages express Cx37; in fact, most are devoid of the 3 vascular connexins. In all 5 mice tested, a strong Cx37 immunostaining was associated with macrophages in the core of advanced atheroscle-

| Cell Type–Specific Connexin Expression at Different Stages of Mouse Atherosclerosis |
|-----------------|-------|-------|-------|
| Cell Type       | Cx37  | Cx40  | Cx43  |
| 0 wk            | ++    | ++    | −     |
| ECs             | −     | −     | +++   |
| SMC media       | −     | −     | +++   |
| 6 wk            | ++    | ++    | −     |
| ECs             | −     | −     | +++   |
| SMC neointima   | −     | −     | ++    |
| SMC media       | −     | −     | ++    |
| Macrophage      | ++    | −     | −     |
| 10 wk           | +     | +     | −     |
| EC shoulder     | −     | −     | +/−   |
| EC center       | −     | −     | −     |
| SMC neointima   | −     | −     | +     |
| SMC media       | −     | −     | +/−   |
| Macrophage      | +     | +     | −     |
| Macrophage core | +/−   | +     | −     |

EC indicates endothelial cells; SMC, smooth muscle cells; +++, abundantly present; ++, present; +, minimally present; +/−, only in some plaques present; and −, absent.
plaques throughout the aorta. The lesions resemble human IDL and LDL particles and development of atherosclerotic cholesterol with a major increase in cholesterol content of experiments with aortas from 4 or 5 mice in each group.

at different stages of mouse atherosclerotic plaques: colocalization of cell markers. Photographs of frozen sections (5 μm) of LDLR−/− mice without dietary intervention (A through D) or after a high-cholesterol diet for 10 weeks (E through H) or 14 weeks (I through L) are shown. Panels A, E, and L show incubation with antibodies against SM-MHC; panels B, F, and J show incubation with antibodies against Cx43; panels C, G, and K show incubation with antibodies against Cx37; and panels D, H, and L show incubation with antibodies against MOMA-2. Bar=125 μm. Similar results were obtained in independent experiments with aortas from 4 or 5 mice in each group.

Figure 4 for a schematic summary).

Figure 3. Connexin distribution in nondiseased mouse aorta and at different stages of mouse atherosclerotic plaques: colocalization with cell markers. Photographs of frozen sections (5 μm) of LDLR−/− mice without dietary intervention (A through D) or after a high-cholesterol diet for 10 weeks (E through H) or 14 weeks (I through L) are shown. Panels A, E, and L show incubation with antibodies against SM-MHC; panels B, F, and J show incubation with antibodies against Cx43; panels C, G, and K show incubation with antibodies against Cx37; and panels D, H, and L show incubation with antibodies against MOMA-2. Bar=125 μm. Similar results were obtained in independent experiments with aortas from 4 or 5 mice in each group.

The pattern of connexin expression in human atherosclerotic plaques was similar to murine expression patterns (online Figures I and II, which can be accessed at http://atvb.ahajournals.org). The results describing connexin expression at different stages of atherosclerotic plaque development are summarized in the Table.

Discussion

We examined the expression of Cx43, Cx40, and Cx37, the 3 major native connexins of the vascular system, during the experimental induction of atherosclerosis. To this end, we studied mice deficient for the LDLR that feature reproducible atherosclerotic plaques after being fed a high-cholesterol diet for 6, 10, or 14 weeks. In the present study, we report a dynamic pattern of vascular connexin expression in atherosclerotic lesions. At last, we compared connexin expression of advanced mouse and human atherosclerotic plaques (see Figure 4 for a schematic summary).

The LDLR removes cholesterol-rich IDL and LDL from plasma, thereby regulating the plasma cholesterol level. Mice lacking the LDLR have plasma cholesterol levels that are 2 times normal but respond to moderate amounts of dietary cholesterol with a major increase in cholesterol content of IDL and LDL particles and development of atherosclerotic plaques throughout the aorta. The lesions resemble human lesions and progress over time from an intimal thickening to a complex lesion with a fibrous cap. Lesson development can be accelerated by a high-fat high-cholesterol diet, as used in the present study.

The endothelium covering the advanced atherosclerotic plaque did not express detectable levels of Cx40 and Cx37. However, these connexins were abundantly expressed in the endothelium next to the plaques and earlier during atherogenesis as well as in the aortic endothelium of control mice, an expression pattern consistent with previous studies involving cows, micropigs, and rats. Furthermore, we observed that Cx43, although absent in control aortic endothelium, appeared in the endothelium at the shoulder region of advanced atherosclerotic plaques, a localization known to experience disturbed hemodynamic forces. In a recent study examining rat aorta and its bifurcation, high levels of Cx43 in the endothelium were associated with areas facing turbulent blood flow. In addition, several in vitro studies show a positive correlation between Cx43 expression and mechanical load or disturbed flow patterns. Taken together, the above-mentioned studies suggest a causal link between endothelial Cx43 expression and hemodynamic conditions that may be relevant to focal vulnerability to atherosclerosis. However, the late stage in mouse atherogenesis, at which we observed the induction of endothelial Cx43 expression, suggests that Cx43 induction may participate in endothelium dysfunction in the late end stage of the disease. Apart from hemodynamic forces, other factors are reported to affect endothelial connexins. For example, age-related downregulation of all 3 endothelial connexins have been described in rat aortas. Treatment with tumor necrosis factor-α, a cytokine that promotes endothelial cell migration, leads to upregulation of Cx43 and downregulation of Cx37 and Cx40 in human umbilical vein endothelial cells, whereas migration of mouse endothelial cells induced by mechanical wounding leads to the upregulation of Cx43 and downregulation of Cx37 but leaves the expression of Cx40 unaffected. In addition, Cx43 and Cx37 in bovine aortic endothelial cells are reported to be differentially regulated by cell density and growth. At last, in regenerating endothelium after denudation injury, the expression of all 3 endothelial connexins initially declines but progressively increases during regeneration although at a different rate for all 3 connexins.
Whether the disappearance of all 3 vascular connexins that we observed in the endothelium covering advanced atherosclerotic plaques participates in endothelial regeneration or migration processes remains to be established.

Adherence of blood monocytes to the dysfunctional endothelium at lesion-prone sites is the earliest observable cellular event in the development of atherosclerosis. Thereafter, monocytes migrate in response to chemotactic stimuli into the subendothelial space, where they undergo proliferation and lipid accumulation, ultimately resulting in the foam cells typical for atherosclerotic lesions. We observed a strong immunolabeling for Cx43 in the core of mouse and human atherosclerotic lesions. This Cx37 immunostaining was colocalized with a marker for macrophages. In addition, we detected a diffuse Cx43 signal throughout the neointima of mouse and human atherosclerotic plaques that may be confined to smooth muscle and inflammatory cells. Indeed, Cx43 mRNA expression has been reported in macrophage foam cells in human and rabbit atherosclerotic plaques. Interestingly, freshly isolated blood monocytes do not express connexin mRNA or protein. Thus, the expression of Cx37 and Cx43 in macrophages in atherosclerotic lesions clearly indicates that gene expression of these gap junction proteins is stimulated on the migration and subsequent differentiation of monocytes/macrophages, suggesting a role for direct cell-to-cell communication in this process.

Cx43 was expressed in different vascular smooth muscular compartments in nondiseased aortas and in atherosclerotic plaques. Whereas Cx43 was the principal gap junction protein present in the media of mouse aortas, an observation consistent with previous studies involving humans, cows, micropigs, and rats, we also observed Cx43 in the neointima of mouse and human atherosclerotic plaques. These observations are in agreement with the hypothesis recently proposed by Ko et al that Cx43 gap junctional intercellular communication in different smooth muscle cell phenotypes may be linked to different functions of smooth muscle cells, such as the ability of smooth muscle cells to coordinate the synthesis of extracellular matrix components, to maintain the functional integrity of elastic media, and/or to regulate the repair and formation of the intima in vascular injury and disease. We observed a trend toward reduced levels of neointimal Cx43 expression with continuing lesion progression in mouse atherosclerotic plaques, an effect that has also been reported during human atherogenesis. Interestingly, we have found that Cx37 was expressed in the media beneath the atherosclerotic plaque, a region in which Cx43 was decreased or often no longer detected. Considering the differential permeabilities of Cx43 and Cx37 gap junction channels, the change in connexin expression in this particular group of smooth muscle cells will certainly lead to a marked change in the pattern of intercellular communication that may affect the function of the smooth muscle cells. An additional connexin isoform, Cx40, has been reported in cultured rat aortic smooth muscle cells, and although this connexin has also been reported in the smooth muscle cells of intact small resistance vessels, it does not appear to be present in the intact mouse aorta.

In summary, we have found that the expression of vascular connexins is differentially regulated during the process of atherosclerosis and that this differential regulation in human plaques largely resembles the one of mice. The change in the distribution of individual connexins over the different compartments of the atherosclerotic plaque suggests that these connexins may differentially regulate the cell-to-cell transfer of factors important for the development of the lesion. Elucidation of how the changes in connexin expression participate in the development of atherosclerotic plaques awaits the availability of novel mouse models in which the expression level of vascular connexins is altered in LDLR−/− mice. At this point, our data indicating that vascular connexins are differentially expressed by atheroma-associated cells within lesions raise the intriguing possibility that apart from paracrine cell-cell communication, direct cell-cell communication via gap junctions may contribute to the still-obscurge pathogenesis of atherosclerosis in humans.

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