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REVIEW

Connexins participate in the initiation and progression of atherosclerosis

Sandrine Morel · Laurent Burnier · Brenda R. Kwak

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Abstract Connexins are members of a large family of transmembrane proteins that form hemichannels or gap junctions. These channels allow the exchange of ions and small metabolites between the cytosol and extracellular space or between neighboring cells. Connexins are important in vascular physiology; they support radial and longitudinal cell-to-cell communication in the vascular wall. Four connexins are expressed in the vascular wall: Cx37, Cx40, Cx43, and Cx45. Their expression is not uniform in all blood vessels and varies with vascular territory and species. Significant changes in the expression pattern of vascular connexins have been described during the development of atherosclerosis, a progressive inflammatory disease. In this review, we provide an overview of (1) the tools used to study the involvement of connexins in atherosclerosis, (2) the participation of connexins in atherogenesis, (3) the increasing interest of a polymorphism in the human connexin37 gene as marker of cardiovascular disease, and (4) the possible therapeutic implications of connexins.

Keywords Connexins · Hemichannels · Gap junctions · Atherosclerosis · Restenosis

Abbreviations

ADP	Adenosine diphosphate
AMI	Acute myocardial infarction
ApoE	Apolipoprotein E
ATP	Adenosine triphosphate
CAC	Carotid artery compliance
CAD	Coronary artery disease
CL	Cytoplasmic loop
CT	COOH-termini
Cx	Connexin
ECs	Endothelial cells
EL	Extracellular loop
ECM	Extracellular matrix
FMD	Flow-mediated dilatation
GFP	Green fluorescent protein
GJIC	Gap junctional intercellular communication
HMG-CoA	3-Hydroxy-3-methylglutaryl-CoA
IMT	Intima-media thickness
LDLR	Low density lipoprotein receptor
MC	Monocytes/macrophages
NT	NH2-termini
PCI	Percutaneous coronary intervention
SMCs	Smooth muscle cells
TNF-alpha	Tumor necrosis factor-alpha

S. Morel · L. Burnier · B. R. Kwak
Division of Cardiology, Department of Internal Medicine,
Geneva University Hospitals, University of Geneva,
Geneva, Switzerland

L. Burnier
Division of Angiology and Hemostasis,
Department of Internal Medicine,
Geneva University Hospitals, University of Geneva,
Geneva, Switzerland

L. Burnier
Service and Central Laboratory of Hematology,
Centre Hospitalier Universitaire Vaudois, University of Lausanne,
Geneva, Switzerland

B. R. Kwak (✉)
Foundation for Medical Research, Division of Cardiology,
Geneva University Hospitals,
64 Av. De la Roseraie,
1211 Geneva 4, Switzerland
e-mail: Brenda.KwakChanson@unige.ch

Introduction

Atherosclerosis is a progressive disease characterized by accumulation of lipids, macrophages, T lymphocytes, and smooth muscle cells (SMCs) in large- and medium-sized arteries. Clinical and experimental observations have led to the notion that the initiating step of this disease is an endothelial dysfunction [1]. This dysfunction leads to an increase in the expression of various cell adhesion molecules and to the secretion of chemoattractants. As a consequence, monocytes transmigrate between endothelial cells (ECs) to infiltrate into the arterial intima where they propagate and mature. These intimal macrophages ingest lipids and transform into macrophage foam cells, forming the earliest atherosclerotic plaque. This initial atherosclerotic lesion is then covered by SMCs that migrate from the media to the intima. In the intima, SMCs proliferate and secrete extracellular matrix (ECM) components that participate to the formation of a strong fibrous cap. In the advanced atherosclerotic plaque, foam cells die and release lipids that form the necrotic core of the lesion. In time, the fibrous cap might rupture inducing the formation of a thrombus at the site of the lesion [2, 3]. This process is implicated in 60% of sudden death by thrombosis [4].

It is now well recognized that inflammation is central in all stages of atherosclerosis [5, 6]. Similar to other inflammatory diseases, paracrine intercellular communication involving cytokines, chemokines, and growth factors is known to play an important role in the development of the atherosclerotic lesions. In this review, we summarize the evidence that another form of intercellular communication involving connexins (Cx) might also be implicated in the development of the disease.

Connexins, connexons, and gap junctions

Connexins

Connexins are members of a family consisting of 20 proteins in mice and 21 in humans. Cx genes are composed of a 5′-untranslated exon, an intron of variable length, an exon harboring the complete coding region, and the 3′-untranslated exon [7]. In some cases, the untranslated exon can be spliced. Two nomenclatures exist to distinguish the different Cx. The first one is based on the molecular mass deduced from their cDNA sequences (for example, the protein with a molecular weight about 43 kDa is called Cx43) The second one is based on sequence similarity and length of the cytoplasmic loop (CL) and separates Cx in four groups: alpha, beta, gamma, and delta (in this system, Cx43 is named “alpha 1” because it has been the first alpha Cx found) [8, 9]. As shown in Fig. 1, a Cx exhibits four α-helical transmembrane domains (M1–M4), two extracellular loops (EL1 and EL2) that are linked by two disulfide bonds, a short CL, and cytoplasmic NH_2- and COOH-termini (NT and CT, respectively). The EL1 and EL2 have highly conserved amino acid sequences and are involved in docking and recognition of compatible Cx [10]. In contrast, the CL is more variable. The CT is characteristic for each Cx; it varies significantly in both length and composition. This domain acts as a substrate for specific kinases or as a partner for other proteins. As a consequence, this domain is involved in the modulation of channel activity in response to appropriate biochemical stimuli [11–14]. Cx work in concert and may have some overlap in function, but the function of one Cx can often not be replaced completely by another Cx isoform [15–17].

Connexons

Cx are synthesized in the endoplasmic reticulum where they form hexameric connexons. This process is completed in the Golgi apparatus after which connexons traffic to the plasma membrane (for reviews, see [10, 18]). The connexon is named homomeric when made of identical Cx and heteromeric when multiple Cx isoforms are involved. During intracellular transit, connexons are associated with microtubules to improve the efficiency of the delivery process [19, 20]. During this process, connexons likely remain in the closed configuration to avoid exchange between cytosol and intracellular compartments.

Once integrated in the plasma membrane, the connexons generally stay in a closed configuration under normal conditions, but they may open upon different stimuli such as removal of extracellular calcium, hypoxic or ischemic stress, mechanical stimulation, and dephosphorylation [21, 22]. These hemichannels allow the passage of ions and small molecules (~1,000 Da) such as ATP or NAD^+ between cytoplasm and extracellular space (Fig. 1). Such exchanges are implicated in regulation of cell volume, in paracrine or autocrine signaling, and activation of survival pathways [23].

Gap junction intercellular channels

Once inserted in plasma membrane, connexons can diffuse laterally and dock with another connexon from a neighboring cell. This association between two connexons occurs via noncovalent interactions between the extracellular loops and permits the formation of a gap junction intercellular channel. The channel is named homotypic if connexons are identical and heterotypic if the two connexons are different. These gap junction channels allow the exchange of ions, small metabolites, second messengers, linear peptides, or small silencing RNA

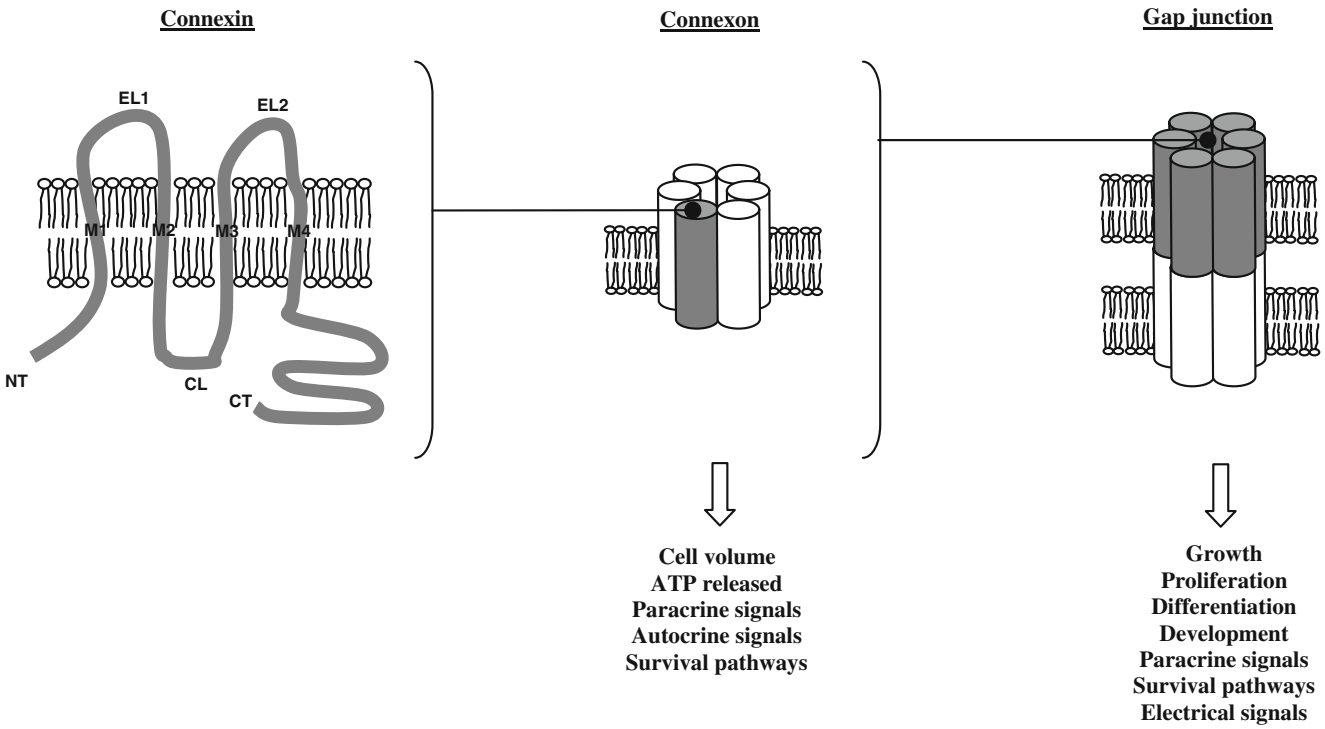

Fig. 1 Schematic representation of connexin topology, a connexon, and a gap junction channel. Connexins have four transmembrane domains (*M1*, *M2*, *M3*, and *M4*), two extracellular loops (*EL1* and *EL2*), a cytoplasmic loop (*CL*), and cytoplasmic NH_2- and COOH-termini (*NT* and *CT*, respectively). Connexons or hemichannels are formed by the association of six connexins. They mediate transmembranous exchange of ions and small metabolites. The associations of connexons from two neighboring cells form gap junction channels. They allow exchange of metabolites or second messengers up to 1-kDa molecular mass between cells in contact

between connected cells [23–25] (Fig. 1). Connexins have a half-lives ranging from 1 to 5 h [25]. Gap junctional intercellular communication (GJIC) allows not only for fast coordinated activities such as contraction of cardiac cells or transmission of neuronal signals at electrical synapses but also for slower physiological processes such as cell growth and development.

Tools to study connexins

Chimeric connexins

Chimeric connexins are Cx tagged at the CT with different compounds like, for example, chemiluminescent aequorin or green fluorescent protein (for reviews, see [10, 26]). These protein reporters induce an increase of the molecular mass of the Cx and may limit the flexibility of the CT. However, these tags do generally not change the trafficking characteristics of the proteins and do also not inhibit the formation of gap junction channels. Chimeric connexins have been used to visualize the intracellular trafficking of Cx on their way to form gap junctions.

Transfected cells

Transfection of cultured cell lines is often used to study gap junction channel characteristics and possible functions of Cx. In general, experiments are realized with communication-incompetent HeLa cells [27], N_2A cells [28], or SKHep1 cells [29] transfected with human or mouse Cx. In these cell lines, specific permeability and charge selectivity of each type of gap junction channel is conserved. In the case of the study of atherosclerosis, Wong et al. [30] performed transfection of H36.12j mouse peritoneal macrophage cell line to prove the implication of Cx37 in monocytic cell adhesion.

Transgenic mice

In vivo studies toward atherosclerosis are currently performed by the use of two well-characterized mouse models: the apolipoprotein E ($ApoE^{-/-}$) knockout mice and the low-density lipoprotein receptor knockout mice ($LDLR^{-/-}$). $ApoE^{-/-}$ mice present high plasma cholesterol concentration (400 to 500 mg/dL) and develop spontaneously foam cell-rich depositions throughout the arterial tree [31]. $LDLR^{-/-}$ mice have a lower increase of plasma

cholesterol level (175 to 225 mg/dL) and they develop only minimal atherosclerotic lesions in aortic roots when fed a normal chow diet [32]. Upon feeding these mouse models with a high-cholesterol diet, they both rapidly develop advanced lesions throughout the vascular tree. Atherosclerosis is also studied in Watanabe heritable hyperlipidemic rabbit or in pigs. However, mice are preferred because they can be interbred with other knockout mice to study the effects of those specific molecules.

To date, 20 Cx have been identified in mice, and 18 Cx-deficient mice exist [33]. An important problem with mice knockout for vascular Cx is that these deletions are often lethal. For example, $Cx43^{-/-}$ and $Cx45^{-/-}$ die in utero or shortly after birth. Cx45 knockout mice display normal vasculogenesis but subsequent transformation into mature vessels is interrupted [34]. Cx43 knockout mice present a swelling and a blockage of the right ventricular outflow tract from the heart that lead to failure in pulmonary gas exchange [35]. As a consequence, heterozygous Cx43 mice have been used to study the implication of Cx43 in atherosclerosis. $Cx43^{+/-}$ mice express 50% of the normal Cx43 level [36]. $Cx40^{-/-}$ mouse are viable but sometimes develop arrhythmias [37, 38]. Indeed, Cx40 is prominent in the atrium and its lack leads to a slow conduction within the atrium increasing the risk for atrial flutter. Furthermore, the absence of Cx40 in the His-Purkinje system may lead to a bundle branch block, preferably in the right bundle. In addition, these mice are hypertensive [39]. $Cx37^{-/-}$ mouse are viable as well and their heart function is normal, but females are infertile [40]. Cx37 and Cx40 are co-expressed in ECs. As a consequence, a double deletion of these Cx induces embryonic lethality in these mice due to an excessive dilation of blood vessels [41]. Gene deletion of Cx in mice is frequently used to study disease processes; however, the absence of one Cx may lead to a decreased or increased expression of another Cx. Cx are differentially expressed in different vascular cell types (see below), and cell-specific Cx deletion may be used to study the implication of the Cx in a particular cell type. For example, the Cre-loxP system under the control of the Tie2 promoter has been used to create mice in which Cx40 or Cx43 has been deleted from the endothelium only [42–44].

Connexin antisense, blocking peptides, and enhancing peptides

Different compounds such as heptanol, octanol, 18α-glycyrrhetinic acid, carbonoxolone, or oleamide are known to inhibit GJIC, but their actions are nonspecific [45–47]. As a consequence, specific peptides to block Cx have been generated. These short peptides have a sequence homology with the conserved extracellular loops of Cx, and they can selectively inhibit the activity of one type of gap junction channels in cells containing multiple Cx [48]. They have a rapid and reversible mode of action, and they are nontoxic for the cells (for review, see [49]). Initially, two blocking peptides have been designed, Gap26 and Gap27, corresponding respectively to the sequence of the first and the second extracellular loop of Cx43 (Fig. 2). These blocking peptides have been used in studies toward gap junctional communication between ECs and SMCs and between ECs and macrophages [50–52]. Derivatives of these first blocking peptides have been created to be more or less specific for Cx37 and Cx40 (Fig. 2). They are efficient in both rodent and human cells, thus reflecting the high degree of amino acid conservation in the extracellular loops. In a study concerning atherosclerosis, blocking peptides have been used to prove the implication of Cx37 in the adhesive property of macrophages [30]. Furthermore, reducing conductivity of Cx43 channels with ^{43}Gap26

	Gap 26	Gap 27
Cx43	VCYD**KS**FPISH**V**R ^{43}Gap 26	SRPTEK**TI**FI**I** ^{43,37}Gap 27
Cx40	VCYD**QA**FPISH**I**R ^{40,37}Gap 26	SRPTEK**NV**FI**V** ^{40}Gap 27
Cx37	VCYD**QA**FPISH**I**R ^{40,37}Gap 26	SRPTEK**TI**FI**I** ^{43,37}Gap 27

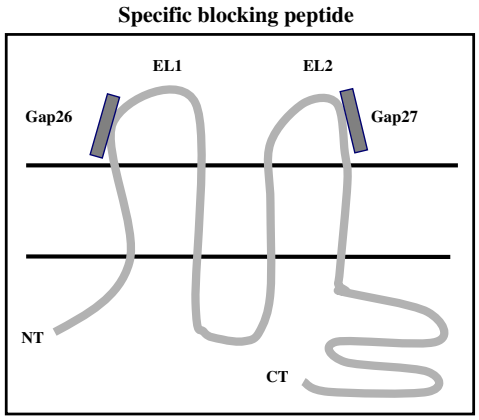

Fig. 2 Connexin-specific blocking peptides. Gap26 and Gap27 sequences correspond, respectively, to the sequence of the first and the second extracellular loops (*EL1* and *EL2*) of connexins. These synthetic peptides inhibit direct intercellular communication in a connexin-specific manner

decreased the adhesion of neutrophils to ECs in vitro and reduced neutrophil recruitment in a mouse model of acute lung inflammation in vivo [53].

There is increasing attention for peptides that selectively open gap junctions. Such peptides might be of particular interest to treat cardiac arrhythmias [54]. More than a decade ago, the first peptide enhancing gap junctional communication (AAP10) has been identified [55, 56]. Stable analogs have been developed since then (ZP123, rotigaptide), although the exact molecular target of AAP10-derived peptides remains to be identified [57]. Moreover, Shibayama et al. [58] identified by phage display a series of RXP peptides capable of binding to the CT of Cx43. One of these peptides, RXP-E, prevents heptanol- and acidosis-induced closure of Cx43 gap junction channels in transfected cells and neonatal cardiomyocytes [58, 59].

The function of connexins in tissues or cells is also investigated using siRNA or antisense oligonucleotides. In experiments toward skin repair, a Cx43 antisense has been prepared in a gel and used in combination with various types of skin lesion. The application of the Cx43 antisense decreased inflammation, lessened scarring, and improved wound closure [60, 61]. In a recent work, we tested Cx43 antisense to inhibit in vitro the dedifferentiation and migration of SMCs, processes implicated in restenosis after ballooning injury [62].

Connexins and gap junctions in healthy vessels

Vascular function is dependent on radial and longitudinal cell-to-cell communication in the vascular wall [63, 64]. It has been extensively reviewed how paracrine molecules such as nitric oxide and prostaglandins secreted by ECs control the vascular tone by their effects on SMCs [65, 66]. In addition, cell-to-cell communication via gap junctions may also be implicated in the control of vascular function [67, 68]. Homomeric and heteromeric channels and homocellular and heterocellular gap junctions are described in the vascular wall (EC–EC, SMC–SMC, EC–SMC gap junctions) [64, 69, 70]. Four Cx are expressed in the vascular wall: Cx37, Cx40, Cx43, and Cx45. Their expression is not uniform in all blood vessels and varies with vascular territory and species. Usually, Cx37 and Cx40 are co-expressed in ECs, whereas Cx43 and Cx45 are present in SMCs (for a review, see [64]). Nevertheless, Cx37 and Cx40 are also found in SMCs of small elastic or resistance arteries or during development [71, 72], and Cx43 is described in ECs at branch points of arteries [73]. The importance of vascular gap junctions has been demonstrated by the fact that Cx deletion alters normal vascular functioning (as described above). Thus, gap junctions interconnecting neighboring ECs allow the spread of signals along the vessel wall, which serve to coordinate vessel behavior. For example, Cx provide the molecular basis for ascending dilatations (i.e., conducted dilations) in arterioles that are required for substantial increases in blood flow during exercise [74, 75]. Among the vascular Cx known to be expressed in ECs, Cx40 appears to play a central role in the arterial conducted response [76, 77]. This is supported by evidence showing that Cx40-deficient mice display impaired conduction of vasodilatation along arterioles in mouse cremaster muscle. Of note, the effect of the absence of one Cx on the expression of other Cx is unclear. In a study on mouse aorta, deletion of Cx40 induced a decrease of Cx37 in ECs, but an increase of Cx37 and Cx43 in the media [78]. In contrast, other investigators reported that deletion of Cx40 was associated with upregulation and redistribution of Cx37 in ECs [79]. Finally, the deletion of Cx37 did not significantly modify the expression of Cx40 in the mouse aortic endothelium [30].

Thus, Cx and gap junctions are central in vascular physiology. In addition, the expression of Cx is modified and implicated in pathological situations such as diabetes, hypertension, or atherosclerosis.

Connexins in atherosclerosis

As mentioned earlier, atherosclerosis is usually studied in mouse deficient in ApoE$^{-/-}$ or for the LDLR$^{-/-}$ [80]. The additional deletion of Cx37, Cx40, and Cx43 in these atherosclerotic-susceptible mice permits to determine the implication of each Cx in atherogenesis. Moreover, the use of the Cre-LoxP system [81] allows for studying the importance of Cx in specific cell types. As shown in Fig. 3, significant changes in the expression pattern of vascular Cx have been described during the formation of atherosclerotic plaques (reviewed in [82]). Moreover, the expression of Cx in vascular wall is influenced by atherosclerotic risk factors, such as turbulent flow, hypertension, and hypercholesterolemia, which act on ECs, on SMC activation, and proliferation and on the inflammatory process (for a review, see [64]). By affecting Cx expression, these risk factors modify gap junction channel- or hemichannel-mediated communication between cells and influence the progression of atherosclerosis.

Connexin37

Cx37 is expressed in healthy ECs, but disappears from these cells in the advanced atherosclerotic plaque [83]. A similar observation has been reported in mice subjected to a high-cholesterol diet for several months [84]. Moreover, Cx37 expression is found in macrophages in early and late atheroma [83, 85]. Taking into account that ECs and

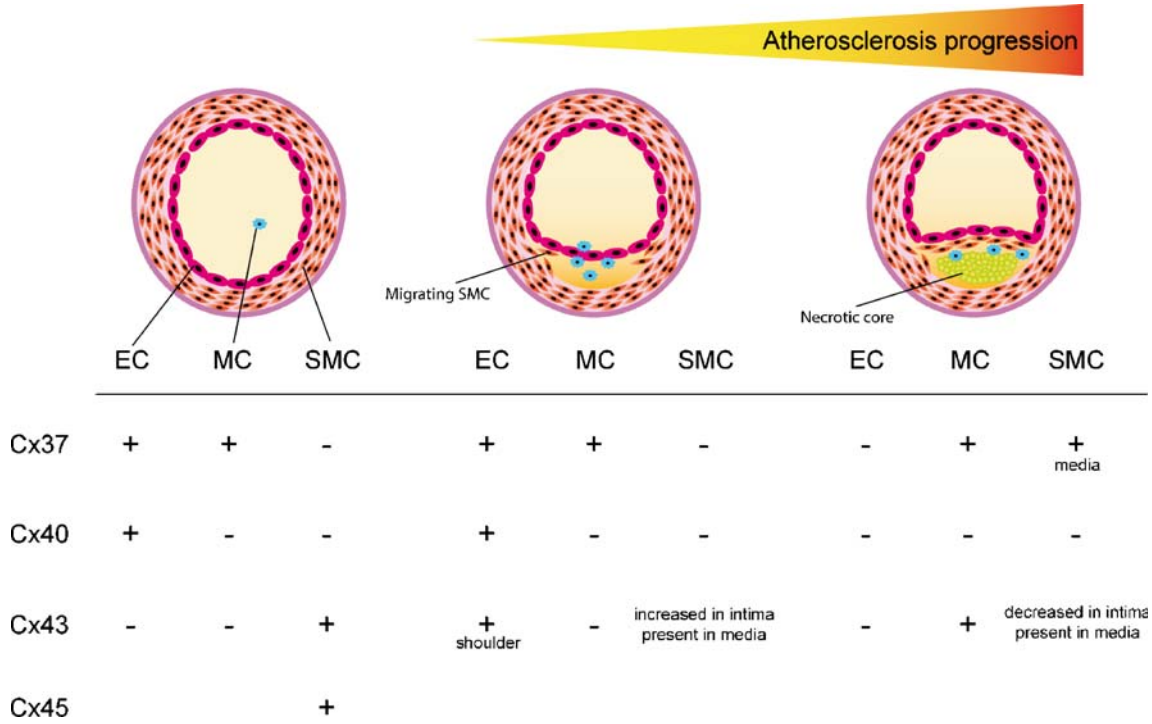

Fig. 3 Evolution of connexin expression during atherosclerosis progression. Atherosclerosis is a progressive vascular pathology implicating endothelial cells (*ECs*), monocytes/macrophages (*MCs*), and smooth muscle cells (*SMCs*). Four connexins are expressed in the vascular wall. Cx37, Cx40, and Cx43 have dynamic expression patterns in healthy vessels and during atherogenesis. Relatively little information is available on Cx45

monocytes/macrophages have central roles in atherogenesis, Cx37 was expected to play a role during atherosclerotic lesion development. Although $Cx37^{-/-}$ mice are infertile due to the absence of ovulation [40], their vascular function is normal [77] and they can be used to study atherosclerosis.

In a study performed in our laboratory, $Cx37^{-/-}$ mice have been crossed with $ApoE^{-/-}$ mice and subjected to a high-cholesterol diet for 10 weeks [30]. The deletion of Cx37 accelerated atherosclerotic lesion development in thoracic–abdominal aorta and in aortic sinus in comparison with control ($Cx37^{+/+}ApoE^{-/-}$) mice. Thus, Cx37 appeared to have a protective effect against atherosclerosis in $ApoE^{-/-}$ mice. The initiating event in atherosclerosis is endothelial dysfunction that leads to monocyte recruitment at the site of the injury in response to chemotactic factors. Monocytes adhere to the ECs, transmigrate across them, and penetrate in the arterial intima where they proliferate, mature, and accumulate lipids to finally progress into macrophage foam cells. As Cx37 is expressed in ECs and monocytes and gap junctions between ECs and leukocytes have been demonstrated (for reviews, see [85, 86]), the role of Cx37 in transmigration was then investigated. For this purpose, fluorescent control and Cx37-deficient monocytes or macrophages were introduced by adoptive transfer in control and Cx37-deficient hypercholesterolemic mice, and the number of fluorescent leukocytes within atherosclerotic plaques was determined [30]. These experiments showed that the deletion of Cx37 in monocytes/macrophages increased the number of leukocytes in atherosclerotic plaques. Interestingly, the presence or the absence of Cx37 in ECs did not influence the transmigration of leukocytes. Thus, the recruitment of leukocytes appeared dependent on the presence of Cx37 in monocytes/macrophages rather than on the existence of gap junction between these cells and ECs, or on intercellular communication within the endothelium. Next, in vitro experiments showed that the deletion of Cx37 in monocytes/macrophages enhanced adhesion of these cells. Similar results were obtained using α-glycyrrhetic acid and connexin blocking peptides. Together, these results demonstrated the implication of functional hemichannels in the adhesion of monocytes/macrophages during atherosclerotic plaque development. Inflammation is mediated in part by extracellular purines (ATP, ADP, adenosine), and ATP is known to pass through various types of gap junctions and hemichannels [87]. The absence of Cx37 or the inhibition of Cx37 by blocking peptides reduced the release of ATP by monocytes/macrophages and increased their adhesion [30]. The use of extracellular ATP scavenger confirmed this result. We therefore proposed that Cx37 protects against atherosclerosis by regulating ATP-dependent monocyte adhesion [30].

Cx37 is also expressed in medial SMCs beneath advanced atherosclerotic lesions in mice [83]. A similar Cx37 expression pattern is observed in advanced atherosclerotic plaques in human carotid artery. The role of Cx37 in these SMCs remains to be established.

Connexin40

Similar to Cx37, Cx40 is present in ECs of healthy vessels, and this Cx disappears from the endothelium covering advanced atherosclerotic plaques [83]. Endothelial Cx40 expression and function is influenced by different factors such as oxidative stress, prothrombotic molecules, pro-inflammatory cytokines, and classic cardiovascular risk factors [88]. Recent studies have shown that abrupt reoxygenation following hypoxia reduces gap junctional coupling between microvascular ECs of wild type but not of Cx40-deficient mice. The reduction in GJIC involves a protein kinase A-dependent pathway and reactive oxygen species [89]. Hyperhomocysteinemia is associated with impaired endothelial-dependent vasodilation and increased risk of atherosclerosis and thrombosis. In a rat model of hyperhomocysteinemia, a downregulation of Cx40 mRNA is described [90]. Tumor necrosis factor alpha (TNF-alpha) is a potent pro-inflammatory cytokine that activates ECs during pathological situations. In human umbilical vein endothelial cells, TNF-alpha treatment decreases Cx40 [91]. Furthermore, a recent study on streptozotocin diabetic mice suggests that downregulation of Cx40 expression and the resultant inhibition of GJIC contribute to coronary vascular dysfunction in diabetes [92].

As mentioned earlier, Cx40-deficient mice are hypertensive. This hypertension is, in part, due to the requirement of Cx40 for longitudinal transmission of endothelium-dependent vasodilator responses [76]. Moreover, blood pressure is controlled by the renin–angiotensin–aldosteron system. In the juxtaglomerular apparatus, Cx40 gap junctions link the ECs of the afferent arteriole to the renin-secreting cells. Two distinct studies have shown that the deletion of Cx40 increases the number of renin-secreting cells and enhances the renal production and release of renin [93, 94]. The role of Cx40 in the renal barosensor mechanism controlling renin synthesis and secretion has been demonstrated with a pharmacological gap junction blocker [94]. The hypertension observed in mice with ubiquitous Cx40 deletion prevents an in vivo study of the implication of Cx40 in atherosclerosis. To avoid this deleterious effect, we have made atherosclerosis-susceptible ApoE$^{-/-}$ mice with specific Cx40 deletion in ECs. Indeed, these mice are not hypertensive and have a normal heart rate [43]. Preliminary data indicate that the EC-specific deletion of Cx40 induced increased atherosclerotic plaque development compared to control mice [43]. These results suggest an atheroprotective role of Cx40, but the mechanisms implicated remain to be investigated.

Connexin43

In healthy vessels, Cx43 is mostly expressed in SMCs. Coronary arteries of hearts removed from patients undergoing cardiac transplantation show markedly increased Cx43 expression in gap junctions between intimal SMCs compared with undiseased vessels [95]. In advanced atherosclerotic plaques, the intimal expression of Cx43 declines. In LDLR$^{-/-}$ mice fed a cholesterol-rich diet, Cx43 increased in intimal SMCs in early atherosclerotic lesions [83]. Cx43 expression was also shown in macrophage foam cells of mouse aorta and of human carotid artery [83, 96], in ECs covering the shoulder region of atherosclerotic lesions [83], and in ECs at branch points of large arteries [73].

Atherosclerotic plaques are generally formed at branch points or at curved areas of large arteries that are regions associated with turbulent blood flow [97]. Oscillatory shear stress induces a high and rapid increase of endothelial Cx43 expression [98]. The effects of unidirectional shear stress on endothelial Cx43 expression are less clear. This shear stress is associated with an increase or with no change in Cx43 expression dependent on the experimental conditions used [98, 99]. Increased hydrostatic pressure does not modify the Cx43 level in ECs [98].

As previously mentioned, Cx43 knockout mice die in utero or shortly after birth [35]. As a consequence, we have studied the implication of Cx43 in the development of the atherosclerotic plaques by interbreeding atherosclerotic-susceptible LDLR$^{-/-}$ mice with heterozygous Cx43$^{+/-}$ mice. The expression of Cx43 was reduced by half in Cx43$^{+/-}$mice [36]. Ten-week-old Cx43$^{+/+}$ LDLR$^{-/-}$ and Cx43$^{+/-}$LDLR$^{-/-}$ mice were fed a cholesterol-rich diet for 14 weeks to evaluate the progression of atherosclerosis. Cx43$^{+/-}$LDLR$^{-/-}$ mice showed reduced atherosclerotic plaque development in the thoracic–abdominal aorta and in the aortic sinus by about 50% in comparison to Cx43$^{+/+}$LDLR$^{-/-}$ mice [100]. Moreover, atherosclerotic lesions in Cx43$^{+/-}$LDLR$^{-/-}$ mice have smaller lipid cores and fewer macrophages, whereas leukocyte counts in peripheral blood were similar between both groups of mice. In addition, the fibrous cap of atherosclerotic plaques in Cx43$^{+/-}$LDLR$^{-/-}$ mice contained more SMCs and interstitial collagen. During the development of the atherosclerotic lesion, SMCs migrate from the media to the intima where they multiply and produce components of the ECM. During this process, SMCs are transformed from the differentiated contractile state to the activated synthetic state. Curiously, synthetic SMCs have been described to express higher levels of Cx43 than the contractile phenotype [62, 101].

The vulnerability of atherosclerotic lesions to rupture is dependent of the content of SMCs and macrophages, the extent of collagen within the lesion and the size of the lipid core. As plaque rupture might lead to acute myocardial infarction, targeting Cx43 may be promising for stabilization of the plaque. Actually, mechanisms by which Cx43 influences atherosclerotic lesion formation and plaque stability are not clearly identified. It has been hypothesized that the effect of Cx43 might depend on specific atheroma-associated cell types [102]. In ECs, Cx43 might induce or enhance endothelial dysfunction. In leukocytes, Cx43 might enhance their migration and proliferation, or might decrease their apoptosis in the atherosclerotic plaque. In SMCs, Cx43 might limit their activation, proliferation, and migration from the media to the intima, or might increase their apoptosis in the plaque. These hypotheses concerning the effects of Cx43 in ECs, leukocytes, and SMCs during atherogenesis are currently investigated in mice with cell-specific deletion of Cx43. Preliminary data showed that endothelial-specific deletion of Cx43 in mice provided beneficial effects on both the natural progression and composition of atherosclerotic lesions [44].

Connexin37 polymorphism and atherosclerosis

Krutovskikh et al. have discovered in 1996 a first polymorphism in the human Cx37 gene while investigating lung and breast carcinoma for mutations [103]. This polymorphism results in an amino acid change at codon 130, which is situated in the cytoplasmic loop of Cx37. Following this first description of a Cx37 gene polymorphism in the human population, Boerma and coworkers [104] have described a second polymorphism in the human Cx37 gene in 1999. This polymorphism corresponds to a cytosine-to-thymine replacement at the position 1019 in the Cx37 gene, resulting in an amino acid alteration in the CT of the protein; a proline residue at position 319 (Cx37-319P) is replaced by a serine residue (Cx37-319S). In this study, authors showed that Cx37-319P was correlated with the occurrence of significant atherosclerotic plaques in carotid arteries in the Swedish population [104]. These results have been confirmed in coronary arteries in other studies performed in Taiwan [105] and Switzerland [106]. In contrast, Collings et al. showed that C1019T polymorphism was not related with markers of subclinical atherosclerosis in young adults in Finland [107]. When myocardial infarction was used as a clinical endpoint, a study in Japanese population showed that Cx37-319S was associated with increased risk in men [108]. This result has been confirmed in a Sicilian population [109]. Discrepant results obtained between the different studies might depend on various reasons such as the chosen clinical endpoint (coronary stenosis versus acute myocardial infarction), the sample size, phenotypic heterogeneity, racial differences, or environment interactions. The different studies concerning Cx37 polymorphism are listed in Table 1. A recent study described the influence of smoking on atherosclerosis in relation with the Cx37-C1019T polymorphism. The authors observed that variation in the Cx37 gene might modify the effects of smoking on the vascular function [110].

As previously mentioned, Cx37 interferes with leukocyte adhesion by releasing ATP. Monocytes transfected with Cx37-319P or Cx37-319S present different adhesion

Table 1 Cx37 polymorphism studies in relation to artery disease and myocardial infarction

Population	Pathology	Cx37 polymorphism prognostic marker	References
Hypertensive Swedish men	Carotid disease	P	[104]
Taiwanese patients receiving coronary catheterization	CAD	P	[105]
Japanese patients with myocardial infarction	MI	S in men No relation in women	[108]
Sicilian young men with acute MI	MI	S	[109]
Irish population with premature onset CAD	CAD, MI	No relation	[118]
American patients with acute coronary syndrome	3-year mortality	S	[119]
Swiss patients requiring angiographic evaluation	CAD	P	[106]
Centenarian Sicilian men	MI	S	[120]
Finnish children and adolescents	IMT, CAC, FMD	No relation	[107]
Northern Han Chinese patients with CAD	CAD	P in men No relation in women	[121]

The most studied polymorphism in the human Cx37 gene corresponds to a cytosine-to-thymine replacement at the position 1019 (Cx37-1019C and Cx37-1019T), which leads to a replacement of proline residue at position 319 (Cx37-319P) in the carboxyl tail by a serine residue (Cx37-319S)

CAD coronary artery disease, *MI* myocardial infarction, *IMT* intima-media thickness, *CAC* carotid artery compliance, *FMD* flow-mediated dilatation

properties and this difference seems due to different ATP permeability [30]. Indeed, Cx37-319P transfected monocytes release more ATP than Cx37-319S transfected monocytes and have lower adhesive properties. These differences may explain the protective effect on acute myocardial infarction conferred by this polymorphic variant. In a larger context, other inflammatory pathologies in which monocytes/macrophages are involved may also be associated with this Cx37 polymorphism. In general, the identification of predisposing genetic factors might help to identify individuals with increased risk for the development of atherosclerosis or other inflammatory pathologies.

Therapeutic implications of connexins in the treatment of atherosclerosis

Regulation of connexin expression by statin treatment

Reduction of atherosclerosis-related morbidity and mortality is possible by lowering plasma cholesterol with statins (inhibitors of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase) [111]. In addition, in vivo and in vitro studies suggest that statins modulate atherogenesis and plaque rupture by mechanisms independent of the decrease of plasma cholesterol concentration [112]. Interestingly, various types of statins dose dependently inhibited Cx43 expression in human vascular cells [100]. In addition, the presence of L-mevalonate abolished the effect of statins on Cx43 expression, confirming that HMG-CoA reductase was responsible for this reduction. The reduction in Cx43 expression was associated with reduction in GJIC. In mice, statin treatment does not reduce plasma lipid levels due to a compensatory upregulation of HMG-CoA reductase. The maintenance of high plasma lipids allows the study of the pleiotropic effects of statins independently of their effects on plasma cholesterol. Statins reduce Cx43 expression in atherosclerotic plaque of $LDLR^{-/-}$ mice and displays beneficial changes in plaque morphology [100]. These observations are comparable to the observations in $Cx43^{+/-}LDLR^{-/-}$ mice. Otherwise, long-term hyperlipidemia in mice decreased Cx37 and Cx40 expression in aorta [84]. Treatment with simvastatin reversed this hyperlipidemia-induced decrease in Cx37 and Cx40. Thus, the statin-induced regulation of Cx expression might be classified as one more pleiotropic beneficial effect of these compounds.

Connexin expression and percutaneous coronary interventions

Coronary atherosclerosis might lead to the occlusion of the artery and to myocardial infarction. This vascular problem is often treated by percutaneous coronary intervention (PCI) consisting of balloon dilatation with or without stent implantation. Clinical studies have shown, however, that the long-term efficacy of PCI is limited by restenosis or renarrowing of the arteries at the site of intervention [113]. Indeed, the stretching of a diseased artery can induce an exaggerated response to injury that involves the recruitment and infiltration of leukocytes into the damaged site and a surge in cytokines and growth factors. Moreover, medial SMCs undergo a phenotypic modulation from a contractile to a synthetic phenotype, proliferate, and migrate toward the intima. Together, these events induce the formation of the neointima. Drug-eluting stents prevent restenosis by inhibiting neointimal hyperplasia. Unfortunately, they also delay re-endothelialization, which increases the period of time during which the stent remains thrombogenic leading to late in-stent thrombosis [114]. Yeh and colleagues have described an upregulation of Cx43 between medial and intimal SMCs after balloon catheter injury in the rat carotid artery [115]. To investigate a possible role of Cx43 in neointima formation, we have performed carotid balloon distension injury in hypercholesterolemic $Cx43^{+/-}LDLR^{-/-}$ mice [116]. This technique induced endothelial denudation and activation of medial SMCs. Neointima formation, macrophage infiltration, SMCs migration, and proliferation were reduced in $Cx43^{+/-}LDLR^{-/-}$ mice, and endothelial repair was accelerated as compared to $Cx43^{+/+}LDLR^{-/-}$ mice. Furthermore, recent in vitro studies showed that Cx43 antisense prevented platelet-derived growth factor-BB-induced deleterious phenotypic changes of porcine SMCs [117]. Together, these results suggest that targeting Cx43 may be a promising strategy for reducing restenosis after PCI. In this respect, recent in vivo applications of Cx43 antisense gel to increase wound healing and to limit burn extension in the mouse skin [60, 61] are of particular interest.

Conclusion

In this review, we provide an overview of the implication of Cx in atherosclerosis and describe pilot work toward possible future therapeutic strategies involving these proteins. The importance of each Cx is revealed by the use of transgenic mice, transfected cells, specific blocking peptides, and antisense. Clearly, further investigations are needed to better understand the exact role of each Cx in the various cell types involved in atherogenesis. Therapeutic targeting of Cx might become promising to limit deleterious consequences of percutaneous coronary interventions. Otherwise, studies toward Cx polymorphisms as marker of cardiovascular diseases is of increasing interest.

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