J Mol Med (2007) 85:787–795
DOI 10.1007/s00109-007-0169-2

REVIEW

Connexin37: a potential modifier gene of inflammatory disease

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Received: 21 December 2006 / Revised: 31 January 2007 / Accepted: 1 February 2007 / Published online: 22 February 2007

Abstract There is an increasing appreciation of the importance of gap junction proteins (connexins) in modulating the severity of inflammatory diseases. Multiple epidemiological gene association studies have detected a link between a single nucleotide polymorphism in the human connexin37 (Cx37) gene and coronary artery disease or myocardial infarction in various populations. This C1019T polymorphism causes a proline-to-serine substitution (P319S) in the regulatory C terminal tail of Cx37, a protein that is expressed in the vascular endothelium as well as in monocytes and macrophages. Indeed, these three cell types are key players in atherogenesis. In the early phases of atherosclerosis, blood monocytes are recruited to the sites of injury in response to chemotactic factors. Monocytes adhere to the dysfunctional endothelium and transmigrate across endothelial cells to penetrate the arterial intima. In the intima, monocytes proliferate, mature, and accumulate lipids to progress into macrophage foam cells. This review focuses on Cx37 and its impact on the cellular and molecular events underlying tissue function, with particular emphasis of the contribution of the C1019T polymorphism in atherosclerosis. We will also discuss evidence for a potential mechanism by which allelic variants of Cx37 are differentially predictive of increased risk for inflammatory diseases.

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Keywords Connexin · Hemichannels · Atherosclerosis · Monocytes · Inflammation · Polymorphism

Connexins, connexons, and gap junctions

Connexins (Cx) are members of a family of proteins encoded by at least 20 different mammalian genes that are expressed in a wide variety of tissues [1, 2]. These genes show 40% sequence identity and a common structure, the

exon being interrupted by introns in only a few exceptions [3]. Accordingly, the amino acid sequences of Cx proteins are highly conserved. A connexin exhibits four α-helical transmembrane domains (M1–M4), two extracellular loops (E1 and E2), a short cytoplasmic loop (CL), and cytoplasmic NH_2- and COOH-termini (NT and CT, respectively). Connexins are classified in three-to-four groups, and the most used nomenclature distinguishes Cx by their molecular mass deduced from their respective cDNAs. The CT, which varies significantly in both length and composition, is nearly unique to each Cx type. For most Cx studied so far, the CT is a substrate for specific kinases and/or protein partners, acting as a regulatory domain to modulate activity of Cx channels in response to appropriate biochemical stimuli [4–6].

The life cycle of connexins begins with the non-covalent oligomerization of 6 Cx monomers into annular structures called connexons [7, 8]. Connexons can be made of one (homomeric) or several (heteromeric) Cx types. After their assembly, connexons are delivered in vesicular carriers traveling along microtubules from the Golgi to the plasma membrane. These connexons at the plasma membrane move laterally to reach the margins of channel clusters and dock with their counterparts in the neighboring cells to form intercellular channels, the gap junctions [9]. Thus, gap junctions grow by accretion at their outer margins from connexons to form plaques that can be resolved by electron microscopy [10].

Connexins, connexons, and gap junctions are involved in numerous processes contributing to the maintenance of normal cell growth and differentiation [1, 11]. Particularly, connexons can function as hemichannels in transmembrane signaling, whereas gap junctions mediate the direct exchange of ions and small molecules (second messengers, metabolites, linear peptides, mRNA) between cells in contact [12, 13]. Experiments of functional replacement of one connexin gene with another have revealed that cellular homeostasis depends on the correct types of Cx expressed [14]. This implies that the specific trafficking, permeability, and interaction with protein partners and transduction networks of each Cx type are contributing to tissue response. Connexons and gap junctions are membrane channels that are gated by chemicals and by membrane potential (V_m). Whereas gap junction channels remain open when V_m is identical between cells (V_m in cell 1 is equal to V_m in cell 2, $V_{m1} = V_{m2}$), they close with increasing differences in transjunctional potential ($V_j = V_{m1} - V_{m2}$). In contrast, hemichannels seem to open with long V_m depolarization [15, 16]. It is therefore not surprising that mutations and polymorphisms of connexin genes would affect Cx-made channel functions and, thus, are associated with a variety of pathological conditions [17]. In this paper, we will review the current knowledge on Cx37 function and discuss evidence for a potential mechanism by which allelic variants of Cx37 are differentially predictive of increased risk for inflammatory diseases.

Specific expression of Cx37 and its role in tissue physiology

Some Cx display a rather ubiquitous expression pattern, whereas others show a more restricted expression to certain organs or cell types where they exert a unique role in tissue function. Cx37, which belongs to the latter group, has been found in the ovary, the vasculature, and inflammatory cells.

Ovary

In the developing ovarian follicle, the oocyte is separated from the local blood supply by an increasing number of granulosa cell layers. These cells, which form the theca externa, are the only ones in direct contact with ovarian capillaries [18]. In this avascular system, intercellular communication via gap junctions between the oocyte and the surrounding somatic cells is essential for correct functioning and development of the follicle [19, 20]. Gap junctions mediate metabolic cooperation between granulosa cells and the oocyte by transmitting endocrine, paracrine, and growth factor effects [21, 22]. Consequently, it has been hypothesized that gap junctional intercellular communication (GJIC) may play a role in the coordination of follicular growth and steroid hormone production [23] as well as in the maturation of the oocyte [24]. Immunohistochemistry has revealed Cx37 in the gap junctions between the oocyte and the granulosa cells of the follicle [25, 26]. In addition, Cx43 has been identified as the major component of gap junctions between granulosa cells. Targeted disruption of the gene encoding Cx37 in mice (Gja4) results in female infertility [25]. In fact, Cx37-deficient mice lack mature Graaf follicles, fail to ovulate, and develop numerous inappropriate corpora lutea. These results suggest that in the normal Cx37-expressing follicle, GJIC allows for bidirectional signaling. On the one hand, the GJIC between the oocyte and surrounding granulose cells are required for oocyte growth and development during the pre-antral stages of the follicle. On the other hand, an inhibitory signal is transferred through gap junctions from the oocyte to the granulosa cells that results in the prevention of luteinization until ovulation has occurred [27]. An additional role that has been proposed for follicular gap junctions is the maintenance of meiotic arrest of the oocyte in a follicle via low tonic amounts of cAMP signaling from the granulosa to the oocyte [24, 28–30].

Blood vessels

The vascular endothelium consists of a continuous monolayer of cells, lining the luminal surface of the entire cardiovascular system, providing a non-thrombogenic barrier between the blood and the underlying tissues. Four connexins, namely Cx37, Cx40, Cx43, and Cx45, have been described in the vascular wall, a tissue that contains not only endothelial–endothelial and smooth muscle–smooth muscle gap junctions but also endothelial–smooth muscle transmembrane channels [31–36]. Although connexin expression profiles have not yet been completely described for all parts of the vascular tree, it is already clear that Cx expression is not uniform in all blood vessels [37]. In addition, differences in Cx expression have been reported in some vessels, like coronary arteries, when comparing different species [38]. Most commonly, endothelial cells (ECs) express Cx37 and Cx40, whereas smooth muscle cells (SMCs) express Cx43 and Cx45. Cx43 has also been found in a subset of ECs near branch points of arteries and in other localizations subjected to oscillatory flow [39, 40]. The replacement of the Cx43 gene by a LacZ reporter gene has revealed the expression of this connexin in ECs of capillaries [41]. Others have reported the expression of Cx37 or Cx40 in SMCs of specific blood vessels [42–44] or under specific conditions [40, 45–47]. Of note, Cx37 might be excluded form myoendothelial junctions, as recently reported in an in vitro model [48].

Several physiological roles have been proposed for vascular gap junctions. Arterioles within the microcirculation span considerable distances, and coordination of cellular behavior is required to allow for the synchronous diameter changes over the entire length of the vessel that are necessary for drastic changes in blood flow. GJIC appeared crucial for the conduction of vasomotor responses along arterioles and small arteries [49–51]. Moreover, ECs are induced to migrate during the process of new capillary sprout formation and during repair of the endothelial lining after injury in large vessels. In a microvascular cell line in which the expression of endothelial Cx was altered by dominant negative connexin inhibitors, wound-induced migration of ECs was found to be dependent on temporary switches in Cx expression [52].

Connexin45, Cx43, Cx40, and Cx37 gene-targeted mice have been created, each having a different vascular phenotype. The complete deletion of Cx45 causes striking abnormalities in vascular development, and mouse embryos die early, between days 9.5 and 10.5 [53]. The deletion of Cx43 causes dramatic cardiac defects, and these homozygous knockout mice ($Cx43^{-/-}$) die in the early postnatal period [54]. To circumvent this problem, the Cre/loxP system was used to inactivate Cx43 expression exclusively in ECs. These conditional Cx43 knockout mice display hypotension and bradycardia [55]. However, this observation remains to be confirmed because similar mice that were developed by another laboratory do not display a vascular phenotype [41]. Although the deletion of Cx37 ($Cx37^{-/-}$) leads to female infertility, these animals survive and do not show an obvious vascular phenotype [25, 56]. The removal of Cx40 ($Cx40^{-/-}$) results in abnormal cardiac conduction [57, 58] as well as in hypertension [59, 60]. More recently, connexin-deficient mice have been interbred to enhance our understanding on the unique and redundant roles of the Cx vascular genes. In contrast to the single knockout animals, mice that completely lack both Cx37 and Cx40 ($Cx37^{-/-}Cx40^{-/-}$ double knockout mice) are not viable beyond the first postnatal day and display severe vascular abnormalities [61]. However, $Cx37^{+/-}Cx40^{-/-}$ mice appeared viable and may be used for studies towards vascular function [62]. In contrast, $Cx43^{+/-}Cx40^{-/-}$ mice exhibit cardiac malformations and die neonatally [63].

Inflammatory cells

The establishment of GJIC between macrophages, based on electrical coupling of adherent murine macrophages, was first reported by Levy et al. [64]. Subsequently, gap junctions were morphologically detected between various types of macrophages and between macrophages and other cell types by freeze fracture electron microscopy [65–68]. Further support for GJIC between macrophages and other cells has come from dye transfer assays. Dye coupling was observed between murine peritoneal macrophages as well as between murine macrophages and intestinal epithelial cells [69]. A low dye coupling was also observed at brain stab wounds and in primary culture of murine microglia [70]. This coupling was dramatically increased with the treatment of IFN-γ and LPS or IFN-γ and TNF-α as well as inhibited by a gap junction blocker. In addition, freshly isolated human monocytes treated with LPS or TNF-α and IFN-γ exhibited dye coupling [71]. However, these studies are in conflict with others reporting lack of GJIC between monocytes/macrophages and other cells. For example, dye transfer was not observed in untreated human or mouse monocytes/macrophages [72, 73], between human monocytes/macrophages and ECs, or between human monocytes/macrophages and SMCs [71, 72].

To date, the expression of two Cxs has been reported in monocytes/macrophages. Cx43 was found in the mouse macrophage cell line J774 [74], activated peritoneal macrophages from hamsters and mice [66, 73, 75], brain stab wound and primary cultures of murine microglia [70], and human monocytes/macrophages stimulated with TNF-α and INF-γ or LPS and INF-γ [71]. Moreover, Cx43 mRNA was detected in macrophage foam cells of human atherosclerotic carotid arteries [72]. In addition, we observed this

connexin in peritoneal macrophages and in macrophages of late atheromas [40, 75]. Finally, Cx37 was also detected in peripheral blood monocytes from human or mice [76]. As described in detail below, Cx37 plays a pivotal role in the recruitment of monocytes and macrophages to atherosclerotic lesions [76].

Epidemiology of Cx37 association with human pathologies

GJIC is often impaired in cancers. When genes coding for Cxs are transfected into cancerous cells, this restores not only their GJIC, but normal growth control is often restored as well [77], thus, identifying connexins as possible 'tumor suppressor genes'. Mutations in Cx proteins can have major effects on GJIC. Interestingly, mutated Cx37 has been reported to be a tumor-associated antigen in the murine Lewis lung carcinoma (3LL-D122) cell line [78]. Moreover, vaccination with a synthetic peptide corresponding to the mutated domain of Cx37 induced effective anti-tumor cytotoxic T lymphocytes and protected mice from spontaneous metastases of 3LL-D122 tumors [79]. In addition, these peptide vaccines reduced metastatic loads in mice carrying pre-established micrometastases [79]. However, genome screening of a set of human lung and breast cancers revealed no somatic mutations in Cx37 in these samples. Interestingly, these studies revealed polymorphisms in the Cx37 gene, but the majority of these polymorphisms reside outside of the open reading frame of the protein [80].

Genetic linkage studies in erythrokeratodermias (EKV), a clinically heterogeneous group of rare autosomal dominant disorders of cornification with hyperkeratosis and erythema, revealed that these diseases map to the chromosomal region 1p34-35 [81]. Human Cx37 gene (GJA4) maps to chromosome 1p35.1 by fluorescence in situ hybridization and was thus considered an attractive candidate gene. By direct sequence analysis of GJA4 in control samples, the authors detected a sequence variant (cytosine-to-thymine) at position 1019, causing a substitution of serine for proline at codon 319 in the regulatory cytoplasmic tail of Cx37. This point mutation creates a unique *Sau* IIIA cleavage sequence that was used to screen all EKV families and a series of unaffected controls for this polymorphism. The serine variant was found in both affected and unaffected EKV family members as well as in a control group of unrelated Caucasians. Moreover, extensive further screening of the EKV families for mutations in GJA4 did not reveal a pathologic sequence aberration in the coding region, thus, excluding Cx37 as a candidate for this disease.

A few years later, a genome-wide linkage analysis for premature myocardial infarction (MI) identified an almost identical region on chromosome 1, i.e., 1p34-36, as novel susceptibility locus for this disease [82]. Coronary artery disease (CAD) is the most common cause of ischemic heart disease resulting primarily from atherosclerosis. The development and outcome of this progressive inflammatory disease are known to depend on the interactions between genetic, behavior, and environmental factors [83]. There are ongoing searches for genes and proteins that influence the development of CAD, with the aim to use these markers along with established risk factors in screening tests for patient risk stratification [84, 85]. These searches have identified genetic polymorphisms in a number of human genes that are associated with CAD and/or MI, including the Cx37 gene.

To date, several gene polymorphism-association studies have detected a link between the C1019T single nucleotide polymorphism (SNP) in the human Cx37 gene and CAD as well as MI in various populations. Surprisingly, the published association studies appear contradictory, which might have arisen in part from comparing different clinical statuses, CAD versus MI. Whereas atherosclerotic plaque development in carotid and coronary arteries seems associated with the 1019C SNP coding for Cx37-319P [86–88], increased risk for MI appeared associated to the 1019T SNP coding for Cx37-319S [89, 90]. This far, only one study could not reveal an association between the C1019T polymorphism in the Cx37 gene and the presence of either CAD or MI [91]. The association between CAD and the Cx37 polymorphism appeared particularly strong in men with type 2 diabetes [92]. In contrast, the polymorphism appeared not associated with other vascular diseases such as hypertension [93] and restenosis after balloon angioplasty [94]. The relevance of Cx37 for MI is further underlined by a report describing an association between this condition and another polymorphism in the 3′-untranslated region of the gene. This I1297D polymorphism may be related to the stability of the mRNA [95].

Although the development of CAD and MI is dependent on many of the same risk factors, the two clinical conditions are considerably different especially regarding features of the atherosclerotic plaques. The key process underlying acute MI is atherothrombosis, which is the rupturing of an unstable or "vulnerable" atherosclerotic plaque followed by acute coronary thrombosis [96, 97]. Plaques that are most likely to break exhibit a thin fibrous cap, a large lipid pool, and many macrophages. This plaque phenotype is partially dependent on the activities of macrophages. Macrophage foam cells secrete pro-inflammatory cytokines that amplify the local inflammatory response in the lesion as well as reactive oxygen species that further induce macrophage proliferation and lipid uptake. In addition, the activated macrophages produce matrix metalloproteinases that can degrade the extracel-

lular matrix, thus, further weakening the plaque's fibrous cap.

Cx37 polymorphism modulates the severity of atherosclerosis: possible mechanisms

The identification of the Cx37 C1019T polymorphism as a prognostic marker for atherosclerosis suggests that sequence differences between the two Cx37 proteins (Cx37-319S and Cx37-319P) account for the phenotype. How can the two forms of Cx37 differently modulate the severity of atherosclerosis? To address this question, we have first evaluated the contribution of Cx37 in the development of atherosclerosis in a mouse model of the disease. Thus, Cx37-deficient mice were crossed with apoliprotein E-deficient ($ApoE^{-/-}$) mice to obtain double knockout animals that were subjected to a high-cholesterol diet [76]. In these mice, the expression of Cx40 was not significantly altered. Deletion of Cx37 accelerated atherogenesis in $Cx37^{-/-}ApoE^{-/-}$ mice as compared to the control group ($Cx37^{+/+}ApoE^{-/-}$). This was demonstrated by the twofold increase of Sudan IV-stained lipids in thoracic abdominal aortas and in aortic sinuses after a 10-week diet. These observations are indicative that Cx37 plays a protective role against atherosclerosis in $ApoE^{-/-}$ mice.

Cx37 is normally expressed in endothelial and macrophage foam cells [40, 98], two cell types that are key players in atherogenesis. In the early phases of atherosclerosis, blood monocytes are recruited to the sites of injury in response to chemotactic factors. Monocytes adhere to the dysfunctional endothelium and transmigrate across ECs to penetrate the arterial intima. In the intima, monocytes proliferate, mature, and accumulate lipids to progress into macrophage foam cells. Because monocytes appeared to express Cx37, the possibility that Cx37 contributes to the interaction between monocytes and endothelial cells was investigated [76]. Indeed, there is evidence in the literature for gap junction-mediated heterocellular communication between leukocytes and ECs [98, 99]. To test for this possibility, Cx37-deficient monocytes or macrophages were introduced in hypercholesterolemic recipient mice by adoptive transfer and the number of adherent leukocytes to or within atherosclerotic plaques determined. This was compared with the number of normal leukocytes introduced to Cx37-deficient recipient mice with atherosclerotic lesions. Interestingly, these experiments revealed that deletion of Cx37 in monocyte/macrophages, but not in ECs, did account for higher number of leukocytes associated with atherosclerotic plaques. These results indicate that heterocellular GJIC does not contribute to the increased recruitment of leukocytes to the atherosclerotic lesions but rather suggest a role of Cx37 in monocytes/macrophage function.

Monocyte migration and accumulation of lipid-filled macrophages are critical events in the progression of atherosclerosis. It is currently unclear why macrophages that enter atherosclerotic lesions do not depart with their lipid loads. During their transmigration across the endothelium, monocytes are subject to profound reorganization of their actin cytoskeleton and plasma membrane receptors and adhesion molecules [100]. These modifications enhanced their adhesion properties and ability to migrate on a substrate. In this context, we observed that adhesion of Cx37-deficient monocyte/macrophages to either EC monolayers, plastic, or glass was enhanced as compared to leukocytes normally expressing Cx37 [76]. The implication of Cx37 in the regulation of monocyte/macrophage adhesion was indicated by that connexin-channel blockers, including α-glycyrrhetinic acid and connexin mimetic peptides, increased leukocyte adhesion, and that expression of Cx37 in a Cx-deficient macrophage cell line decreased its adhesiveness to substrates. Because these assays were performed using isolated leukocytes, it is likely that connexons, and not gap junctions, are involved in the process of cell adhesion.

Extracellular purines (ATP, ADP, adenosine) are important signaling molecules that mediate both inflammatory and anti-inflammatory effects. ATP is also known to pass through various types of gap junctions and hemichannels [101]. Interestingly, a causal relationship was observed between extracellular ATP release and decreased adhesion in monocyte/macrophages expressing Cx37. Conversely, absence of Cx37 or blockade of Cx37 hemichannels reduced the release of ATP out of the cells and increased their adhesion to substrates. Furthermore, the use of an extracellular ATP scavenger increased adhesion of normal monocyte/macrophages, whereas addition of extracellular ATP equalized the adhesive properties of Cx37-deficient leukocytes to that of Cx37-expressing leukocytes. Altogether, these observations suggest that extracellular ATP provides a link between Cx37 hemichannel activity and leukocyte adhesiveness. It is hypothesized that Cx37 hemichannels release ATP, which in turn interferes with leukocyte adhesion by a mechanism that remains to be demonstrated (Fig. 1). According to this hypothesis, absence of Cx37 would be associated with increased adhesion of monocyte/macrophages to and within the atherosclerotic plaques. A change in the adhesion properties of these cells will also likely favor their accumulation in the atherosclerotic lesions and worsen the phenotype. In this context, the observation that expression of Cx37-319S or Cx37-319P by transfection of a human macrophage cell line revealed differential adhesiveness to substrates is of particular importance [76]. This may be caused by increased permeability of the Cx37-319P hemichannels for ATP, thus, providing a potential mechanism by which the Cx37-1019C variant protects against atherosclerosis.

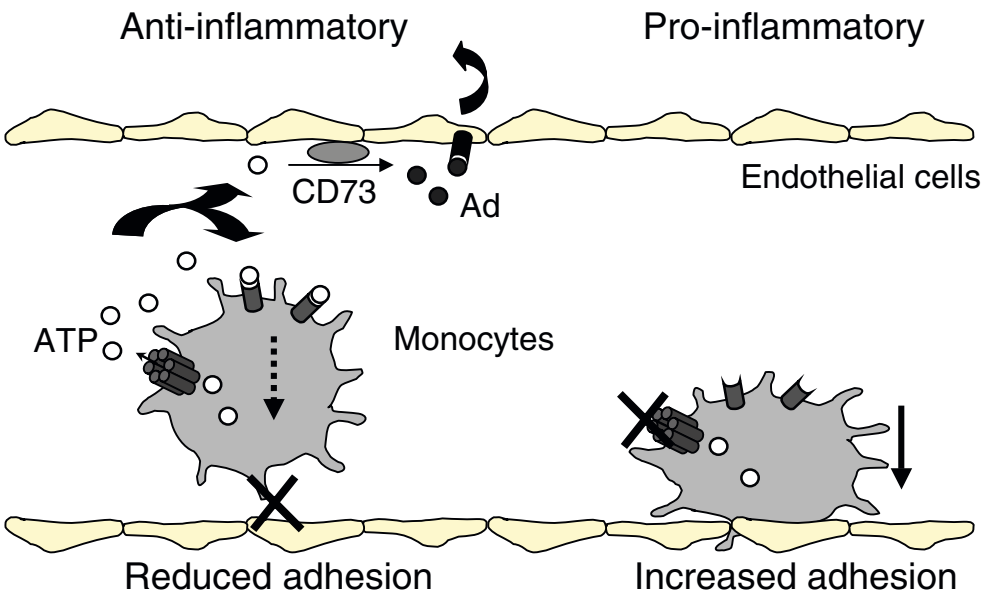

Fig. 1 Hypothetical model of the anti-adhesive function of Cx37 in mouse monocytes. Rolling monocytes at the surface of the vessel slow down and firmly adhere to ECs before extravasation. Cx37-hemichannels at the surface of monocytes allow for the release to the extracellular space of ATP. Extracellular ATP negatively regulates the adhesion of monocytes to ECs by a yet undetermined mechanism. In the absence of functional Cx37 (by hemichannel blockade or Cx37 gene deletion), ATP is not released out of the cell, resulting in enhanced adhesiveness of monocytes to the endothelium. Possibly, ATP released by monocytes can be sequentially degraded by ectoenzymes to AMP and then nucleosides and inosine. For instance, ecto-5′ -nucleotidase (CD73) is up-regulated at the endothelium surface during inflammation to convert AMP into adenosine (Ad). Adenosine has an anti-inflammatory and cell-protective effect through its binding to receptors localized on the cell surface of endothelial and some inflammatory cells [108]. Absence of production of ATP by Cx37-deficient monocytes may reduce adenosine production, which in turn would accelerate atherogenesis in mice by favoring a pro-inflammatory environment

Speculative remarks and conclusion

Additional experiments are needed to determine whether Cx37-319S and Cx37-319P hemichannels exhibit differential biophysical and permeability properties. However, one can speculate on the mechanism underlying the regulation of Cx37-319S and Cx37-319P hemichannels. One consequence of the study by Wong et al. [76] is that leukocytes may need to close Cx37 hemichannels to increase their adhesive properties. It is long known that adherent macrophages showed a more negative membrane potential as compared to macrophages in suspension [102–104]. One consequence of more negative V_m would be to turn off hemichannel activity. Thus, differences in V_m sensitivity between Cx37-319S and Cx37-319P hemichannels could account for the differential ATP transport by these connexons. An alternative possibility is that elevated macrophage plasma membrane cholesterol content may differentially affect the regulation of Cx37-319S and Cx37-319P hemichannels. There is indeed increasing evidence that high cholesterol levels may alter plasma membrane and actin cytoskeleton organization of macrophages during atherosclerosis [105]. The presence of cholesterol in plasma membranes is also known to affect the chemical regulation of gap junction channels [106, 107]. Thus, a differential sensitivity of Cx37-319S and Cx37-319P to cholesterol increase may also account for the enhanced ATP leakage through Cx37-319P hemichannels. Hence, Cx37-319P, by releasing ATP, may reduce the adhesion of macrophages and allow them to egress from the affected area. The decreased adhesiveness of Cx37-319P-expressing leukocytes may therefore serve as a “protector” mechanism that prevents excessive monocyte recruitment in atherosclerosis. Because the rupture of vulnerable atherosclerotic plaques, a key process underlying acute MI, strongly depends on the presence and the activity of macrophages in the lesions, our study may provide a rationale for the epidemiological association between increased risk for acute MI and the Cx37-319S polymorphism. The generation of knock-in mice for either Cx37 polymorph may help to resolve these issues. Our improved understanding of the role of the Cx37 C1019T polymorphism may not only lead to the use of this genetic variant in risk stratification for MI, but may also have implications for other chronic inflammatory diseases where monocytes and/or macrophages are involved.

Acknowledgments We thank Suzanne Duperret for secretarial help. This work was supported by grants from the Swiss National Science Foundation (310000-107846/1 to MC; PPOOA-68883 and 3100-067777 to BRK).

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