Endothelial-Specific Deletion of Connexin40 Promotes Atherosclerosis by Increasing CD73-Dependent Leukocyte Adhesion

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Background—Endothelial dysfunction is the initiating event of atherosclerosis. The expression of connexin40 (Cx40), an endothelial gap junction protein, is decreased during atherogenesis. In the present report, we sought to determine whether Cx40 contributes to the development of the disease.

Methods and Results—Mice with ubiquitous deletion of Cx40 are hypertensive, a risk factor for atherosclerosis. Consequently, we generated atherosclerosis-susceptible mice with endothelial-specific deletion of Cx40 (Cx40del mice). Cx40del mice were indeed not hypertensive. The progression of atherosclerosis was increased in Cx40del mice after 5 and 10 weeks of a high-cholesterol diet, and spontaneous lesions were observed in the aortic sinuses of young mice without such a diet. These lesions showed monocyte infiltration into the intima, increased expression of vascular cell adhesion molecule-1, and decreased expression of the ecto-enzyme CD73 in the endothelium. The proinflammatory phenotype of Cx40del mice was confirmed in another model of induced leukocyte recruitment from the lung microcirculation. Endothelial CD73 is known to induce antiadhesion signaling via the production of adenosine. We found that reducing Cx40 expression in vitro with small interfering RNA or antisense decreased CD73 expression and activity and increased leukocyte adhesion to mouse endothelial cells. These effects were reversed by an adenosine receptor agonist.

Conclusions—Cx40-mediated gap junctional communication contributes to a quiescent nonactivated endothelium by propagating adenosine-evoked antiinflammatory signals between endothelial cells. Alteration in this mechanism by targeting Cx40 promotes leukocyte adhesion to the endothelium, thus accelerating atherosclerosis.

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Key Words: atherosclerosis ▪ connexins ▪ endothelium ▪ gap junctions ▪ inflammation

Cardiovascular diseases currently constitute the major cause of death in developed countries. Atherosclerosis, an inflammatory disease of large and medium-sized arteries, is the most important cause of cardiovascular diseases. The main consequences of atherosclerosis are myocardial infarction, cerebral infarction, and aortic aneurysm.

Clinical Perspective on p 131

Atherosclerosis involves the formation of intimal lesions that are characterized by a dysfunctional endothelium, inflammation, lipid accumulation, cell death, and fibrosis. The distribution of atherosclerotic plaques is highly characteristic in humans; the lesions develop predominantly near side branches of arteries where blood flow is disturbed. A variety of substances mediating intercellular communication, including cytokines, chemokines, and growth factors, have been identified to induce, amplify, and modify the atherosclerotic inflammatory process. In this context, connexins, a large family of proteins that form hemichannels and gap junction channels enabling transmembrane and intercellular coordination of tissue activity, have been involved in atherogenesis. Three connexins are expressed in the vascular wall, namely connxin (Cx)37, Cx40, and Cx43, and important changes in their expression pattern have been reported in...
human vessels and in animal models of atherosclerosis.\(^9\)
Progression of atherosclerosis is reduced in atherosclerosis-susceptible mouse models with genetically reduced Cx43 expression. Atherosclerotic lesions in these mice were less complex, as shown by a reduction in the number of inflammatory cells and a thicker fibrous cap with high collagen content and large numbers of smooth muscle cells, suggesting a more stable plaque phenotype.\(^10\) In contrast to the proatherogenic role of Cx43, Cx37 is atheroprotective. We have recently demonstrated in a mouse model that Cx37 hemichannels control the initiation of atherosclerosis by inhibiting autocrine ATP-dependent regulation of monocyte adhesion.\(^11\) These results are consistent with the increasingly recognized view that connexins are modulators of leukocyte trafficking into inflamed tissues.\(^12\)\(^-\)\(^14\)

In an earlier study, we showed in mice that the endothelium covering advanced atherosclerotic lesions no longer expresses Cx40, whereas this protein is abundantly detected in unaffected endothelium adjacent to the lesions.\(^6\) Cx40 has also been found in healthy endothelium of other species.\(^15\)\(^-\)\(^16\)

The use of Cx40-deficient mice to investigate atherogenesis has been hampered by the report that these animals are hypertensive,\(^17\)\(^-\)\(^18\) a well-known independent risk factor of the disease. To address specifically the role of Cx40 in atherosclerosis, we have now taken advantage of the Cre-LoxP system to create an atherosclerosis-susceptible mouse line in which Cx40 is deleted only from the endothelium.

**Methods**

**Mice**

Generation of the Cx40\(^{fl/fl}\) mice, transgenic mice expressing ubiquitously enhanced green fluorescent protein (EGFP), and mice in which EGFP expression is under the control of the Cx40 gene (Cx40\(^{EGFP}\) mice) is described in Methods in the online-only Data Supplement.

**Experimental Interventions on Mice**

Protocols for induction and quantification of atherosclerosis, measurement of heart rates and arterial pressure by telemetry, and vasomotor responses in isolated aortas are described in Methods in the online-only Data Supplement. Alveolar recruitment of neutrophils was measured after intratracheal instillation of Pseudomonas aeruginosa lipopolysaccharide and peritoneal macrophages were obtained as described in Methods in the online-only Data Supplement.

**Western Blotting and Immunohistochemistry**

Western blotting and immunostaining were performed as described.\(^9\)\(^,\)\(^10\) See also Methods in the online-only Data Supplement.

**Dye Coupling**

Gap junctional intercellular communication (GJIC) was determined by Lucifer yellow transfer assays, as described previously.\(^10\)

**Adhesion Assays**

Adhesion assays were performed with the use of monolayers of bEnd.3 cells and mononuclear H36.12 or THP-1 cells\(^11\) or neutrophils collected by bronchoalveolar lavages (BALs), as described in Methods in the online-only Data Supplement.

**RNA Silencing, RNA Antisense, and Reverse Transcription Polymerase Chain Reaction**

bEnd.3 cell monolayers were transfected for 12 hours with small interfering RNA (siRNA) for Cx40, Cx26, or CD73 with the use of lipofectamine (Invitrogen). In some experiments, bEnd.3 cells were preincubated for 24 hours with 10 \(\mu\)mol/L of Cx40 sense or antisense phosphorothioated oligonucleotides. For details, see Methods in the online-only Data Supplement.

**CD73 Activity**

CD73 activity was determined by release of inorganic orthophosphate, as described in Methods in the online-only Data Supplement.

**Cell Treatments**

CD73 expression and activity were first increased by incubating bEnd.3 cells with 100 \(\mu\)mol/L of the methotrexate analogue aminopterin for 12 hours in culture medium to enhance adenosine release.\(^19\) After aminopterin stimulation, bEnd.3 cells were exposed for 3 hours with the CD73 inhibitor \(\alpha\beta\)-methyleneadenosine 5\'-diphosphate (AMP-CP) (50 \(\mu\)mol/L), the A\(_2\)B receptor antagonist alloxazine (10 \(\mu\)mol/L), or the A\(_2\)B receptor agonist 5\'-N-ethylcarboxamidoadenosine (NECA) (10 \(\mu\)mol/L) alone or in combination. All agents were purchased from Sigma.

**Statistical Analysis**

GraphPad Prism software (version 4.03) was used to compare experiments using paired or unpaired \(t\) tests and the nonparametric Mann-Whitney \(U\) test, where appropriate. Values are expressed as mean±SEM. 

**Results**

**Generation of Atherosclerosis-Susceptible Mice With Endothelial-Specific Deletion of Cx40**

Cx40 is widely expressed in the mouse cardiovascular system; it has been found in endothelial cells (ECs) throughout the vascular tree, smooth muscle cells of small resistance vessels, renal juxtaglomerular cells, and atrial cardiomyocytes. To avoid the hypertension reported with ubiquitous gene deletion of Cx40, we generated mice with a conditional mutation in the Cx40 gene (see Methods and Figure 1 in the online-only Data Supplement for details). We interbred Cx40\(^{fl/fl}\) mice with mice harboring the Cre recombinase coding sequence under the control of the endothelial-specific Tie2 promoter (Tie2\(^{Cre}\)) and atherosclerosis-susceptible apolipoprotein E (ApoE)−/− mice to generate mice with a conditional mutation in the Cx40 gene (see Methods and Figure 1 in the online-only Data Supplement for details). We interbred Cx40\(^{fl/fl}\) mice with mice harboring the Cre recombinase coding sequence under the control of the endothelial-specific Tie2 promoter (Tie2\(^{Cre}\)) and atherosclerosis-susceptible apolipoprotein E (ApoE)−/− mice to generate Tie2\(^{Cre}\) Cx40\(^{fl/fl}\) mice, Tie2\(^{Cre}\) Cx40\(^{fl/fl}\) mice, and Tie2\(^{Cre}\) ApoE\(^{−/−}\) mice (Cx40del) and control Tie2\(^{Cre}\) ApoE\(^{−/−}\) (C1) and Cx40\(^{fl/fl}\) ApoE\(^{−/−}\) (C2) mice.

We determined Cx40 expression in aortic endothelium, blood vessels in the heart, atrial myocardium, and lung capillaries from Cx40del mice and controls by fluorescence microscopy (Figure 1). Cx40 was detected as a typical punctate gap junction staining between neighboring ECs in both control groups (Figure 1A, 1B, 1D, 1E, 1G, and 1H). As expected, Cx40del mice did not express Cx40 between ECs, both in en face staining and in cryosections (Figure 1C, 1F, and 1I). In contrast, we found Cx40 between atrial cardiomyocytes in all 3 groups (Figure 1J to 1L), illustrating that Cx40 was specifically deleted from the endothelium only in Cx40del mice. These results were confirmed by Western blot (Figure 1M). Whereas atrial Cx43 expression was not affected by deletion of Cx40, Cx37 expression was decreased \((P<0.05)\) in Cx40del mice (Figure 1N), as reported previously for mice with ubiquitous deletion of the Cx40 gene.\(^20\)

**Mice With Endothelial-Specific Deletion of Cx40 Have Normal Hemodynamic Parameters**

Blood pressure measurements were performed by telemetry in conscious mice. Four days of monitoring revealed no
significant differences in mean arterial pressure or heart rate between controls and Cx40del mice (Figure 2A and 2B). Moreover, contraction in response to KCl or norepinephrine was not significantly different in aortic rings from controls and Cx40del mice (Figure IIA and IIB in the online-only Data Supplement). Likewise, endothelium-dependent (acetylcholine) and -independent (sodium nitroprusside) relaxations were comparable between the 3 groups (Figure IIC and IID in the online-only Data Supplement). Thus, Cx40del mice display normal aortic endothelium-dependent vasomotor responses and are not hypertensive.

Mice With Endothelial-Specific Deletion of Cx40 Show Accelerated Atherosclerosis

Ten-week-old control and Cx40del mice were fed a high-cholesterol diet for 5 or 10 weeks to induce atherosclerosis. Aortas were stained for lipids with Sudan IV, an indicator for disease progression. After 10 weeks of diet, we observed an increase of lipid staining in the thoracoabdominal aortas of Cx40del mice (21.4 ± 2.5%; n = 10; P < 0.01) compared with both C1 (12.6 ± 1.3%; n = 10) and C2 (7.1 ± 1.3%; n = 10) mice (Figure 3A). There was, however, no significant difference in Sudan IV staining in aortic sinuses from Cx40del (29.1 ± 6.7%; n = 10), C1 (34.9 ± 7.5%; n = 10), and C2 (24.9 ± 6.2%; n = 10) mice (Figure 3B). As expected, fewer and smaller lipid lesions were observed throughout the aortas after 5 weeks of a cholesterol-rich diet. Increased lipid staining was detected in the
thoracoabdominal aortas of Cx40del mice (5.2±0.6%; n=5; P<0.03) compared with C1 (2.9±0.6%; n=5) and C2 (3.0±0.6%; n=5) mice (Figure 3C). Similarly, only Cx40del mice showed lipid staining in the aortic arches at this time. In contrast, no difference in atherogenesis were observed in the aortic sinuses (Cx40del, 21.4±8.3%; C1, 15.3±4.1%; C2, 12.2±3.8%; n=5 per group) (Figure IIIA and IIIB in the online-only Data Supplement). Probably as a result of a combined action of high cholesterol and disturbed blood flow, atherosclerotic lesions first appear in aortic sinuses of ApoE−/− mice.21 As a consequence, differences in atherogenesis due to genes or cell types involved in the initiation phase of the disease might be flattened out in advanced lesions after 5 or 10 weeks of diet. In the absence of diet, there was very little lipid deposition in aortic sinuses of 10-week-old C1 (0.5±0.1%; n=3) or C2 (1.7±0.8%; n=3) mice. In contrast, Cx40del mice showed considerable (P<0.05) lipid deposition at this location (6.4±1.7%; n=3; Figure 3D). These results demonstrate the importance of endothelial Cx40 during early stages of atherosclerosis.

Mice With Endothelial-Specific Deletion of Cx40 Showed Enhanced Monocyte Recruitment

Leukocyte recruitment in early atherogenesis involves mostly monocytes. These cells adhere to the dysfunctional endothelium and then transmigrate across intact ECs to penetrate into the arterial intima, where they proliferate, mature into macrophages, and accumulate lipids.3 We verified the presence of macrophages in atherosclerotic lesions by CD68 staining. No significant differences in CD68 staining were detected in aortic sinuses of Cx40del mice (50.1±6.6%; n=5) after 5 weeks of high-cholesterol diet compared with C1 (36.4±4.4%; n=5) and C2 (41.0±5.4%; n=5) controls. CD68 staining, however, was prominent in aortic sinuses of 10-week-old Cx40del mice without diet, whereas virtually no macrophage infiltration was observed in control mice (Figure 4A to 4C). Vascular cell adhesion molecule-1 (VCAM-1) is a ligand expressed by activated ECs and involved in monocyte adhesion.14 We detected intense VCAM-1 immunostaining along the endothelium in aortic sinuses of Cx40del mice (arrows in Figure 4F), whereas no VCAM-1 staining could be detected in the endothelium of control mice (Figure 4D and 4E). VCAM-1 expression is known to be regulated by the activity of the 5′-ecto-nucleotidase (CD73) at the surface of ECs.22 En face staining in aortas revealed that expression of CD73 was decreased in Cx40del mice compared with controls (Figure 4G to 4I).
CD73 activity in lung microvessels of Cx40del mice was also decreased (Figure 5B). To provide a quantitative assessment of inflammatory cell recruitment in vivo, we evaluated the number of leukocytes that migrated to the alveolar space in response to intratracheal instillation of lipopolysaccharide. As shown in Figure 5C and 5D, the total number of cells detected in the alveolar space, which were mostly neutrophils, was markedly enhanced in Cx40del mice. At these time points, the expressions of Cx37 and Cx40 were not affected by the treatment (Figure 5A). Thus, endothelial deletion of Cx40 is associated with an early proinflammatory phenotype affecting not only monocyte infiltration in atherosclerosis but also the transmigration of neutrophils in a mouse model of acute lung inflammation. Collectively, these results suggest that endothelial Cx40 may intersect with CD73-dependent signaling to regulate leukocyte recruitment.

Deletion of Cx40 in Endothelial Cells but Not Leukocytes Contributes to the Inflammatory State

The Tie2 promoter used here is also active in hematopoietic cells, and the presence of Cx40 in leukocytes is still debated. Cx40 was not detected by Western blots and immunofluorescence in macrophages and neutrophils from wild-type mice (not shown). To definitely address whether Cx40 is expressed in macrophages or neutrophils, we isolated these cells from heterozygous mice in which one of the Cx40 genes was replaced by the green fluorescent protein (Cx40EGFP mice). In contrast to leukocytes from EGFP reporter mice (Figure IVA and IVD in the online-only Data Supplement), we did not detect EGFP fluorescence in neutrophils (Figure IVB and IVC in the online-only Data Supplement), we did not detect EGFP fluorescence in these leukocytes was confirmed by Western blotting (Figure IVG and IVH in the online-only Data Supplement).

CX40 activity and Leukocyte Adhesion In Vitro

The activity of CD73 at the surface of ECs is known to decrease leukocyte adhesion to the endothelium. This antiinflammatory role is mediated by the generation of cAMP after stimulation of A2B receptors by adenosine, which results from the hydrolysis of adenine nucleotides by CD73. As shown in Figure 6A and 6B, aminopterin treatment increased the expression of CD73 in bEnd.3 cells pretreated with amiloride (ALX), or targeting CD73 with a specific siRNA increased the adhesion of neutrophils to ECs. The latter effect was reversed by global activation of A2B receptors with NECA. n=4. *P<0.05 in A and B and P<0.001 in C.

lack of EGFP in expression in these leukocytes was confirmed by Western blotting (Figure IVG and IVH in the online-only Data Supplement).

Targeting Endothelial Cx40 Affects CD73 Activity and Leukocyte Adhesion In Vitro

The activity of CD73 at the surface of ECs is known to decrease leukocyte adhesion to the endothelium. This antiinflammatory role is mediated by the generation of cAMP after stimulation of A2B receptors by adenosine, which results from the hydrolysis of adenine nucleotides by CD73. To investigate the relationship between Cx40 and CD73 in detail, we searched for an appropriate in vitro cell model expressing stable levels of CD73 and Cx40 in culture. Cx40 expression progressively decreases in primary ECs of human and mouse origin with passages, with virtually no remaining protein at passages 3 to 4. In contrast, the mouse bEnd.3 EC line constitutively expresses CD73 and Cx40 properly localized at cell-cell contacts. The methotrexate analogue aminopterin is known to promote adenine nucleotide release and thus the production of adenosine via CD73 activity. As shown in Figure 6A and 6B, aminopterin increased the expression (P<0.05) and surface activity (P<0.05) of CD73 in bEnd.3 cells, an effect that was also
induced by direct activation of A$_2$B receptors by the agonist NECA ($P<0.001$). Conversely, aminopterin-induced CD73 activity was decreased ($P<0.05$) by the inhibitor AMP-CP or after targeting CD73 with a specific siRNA (Figure 6B). Finally, we confirmed that conditions reducing CD73 activity (AMP-CP) or expression (siCD73) enhanced ($P<0.001$) neutrophil adhesion to bEnd.3 cells (Figure 6C). The latter effect was reversed by global activation of A$_2$B receptors with NECA (Figure 6C). Moreover, A$_2$B receptor blocking with the antagonist alloxazine increased ($P<0.001$) adhesion of neutrophils onto bEnd.3 cells pretreated with aminopterin (Figure 6C). Altogether, these experiments define bEnd.3 cells as an appropriate model to further investigate the possible relationship between CD73 and Cx40.

We next developed strategies to inhibit Cx40 expression in bEnd.3 cells. The ability of Cx40 siRNA (siCx40) and antisense Cx40 (ASCx40) to reduce functional expression of Cx40 in bEnd.3 cells was verified by immunofluorescence, reverse-transcription polymerase chain reaction (Figure 7A to 7E), and dye coupling (Figure 7G). Interestingly, ASCx40 and siCx40 decreased CD73 expression and activity ($P<0.01$). Control oligonucleotides, such as sense Cx40 (SCx40) or siRNA targeted against Cx26 (siCx26), which is not expressed by ECs, had no effect. Finally, we studied adhesion of neutrophils and mononuclear H36.12j cells$^{11}$ to bEnd.3 cell monolayers. We found that targeting Cx40 expression in bEnd.3 cells with siCx40 or ASCx40 increased ($P<0.05$) adhesion of both types of leukocytes to the EC monolayer (Figure 7I and 7J). Control siCx26 or SCx40 had no effect on leukocyte adhesion. Direct activation of A$_2$B receptors with NECA reversed the number of adherent neutrophils to control values even in the presence of the siCx40 (Figure 7J). These in vitro results confirm that limiting Cx40 expression affects CD73 expression and activity and thus leads to increased adhesion of leukocytes to the endothelium.

**Cx40 Intercellular Channels Convey Antiadhesion Signals for Leukocytes In Vitro**

To further understand the interaction between Cx40 and CD73, we evaluated whether signals generated by the CD73 pathway affect Cx40 intercellular channel function. To this end, we performed dye coupling in bEnd.3 cells exposed to NECA, aminopterin, or aminopterin+AMP-CP. Interestingly, increasing CD73 activity or direct stimulation of A$_2$B receptors enhanced GJIC, whereas blocking Cx40 decreased GJIC (Figure 8A and 8B). These results suggest that CD73-dependent activation of adenosine receptors will lead to increased GJIC, a process that may favor the cell-to-cell propagation of antiinflammatory signals. To test this, we performed cocultures of Hela cells stably transfected or not with Cx40. As depicted in Figure 8C, Cx40-expressing (Cx40) or wild-type Hela cells were seeded on the bottom side of Transwell filters, whereas Cx40-expressing Hela cells were plated onto the top side. NECA was then applied to the cells below, and adhesion of THP-1 monocytic cells onto Cx40-expressing cells on the top side was determined. We found that THP-1 cell adhesion was decreased ($P<0.001$) in the Cx40/Cx40 combination compared with the wild-type/Cx40 combination, an effect that was reversed in the presence of a gap junction blocker (Figure 8C). Altogether, these results indicate that specific antiadhesion signals generated by the CD73/adenosine system are communicated via Cx40 gap junction channels.

**Discussion**

Previous studies have shown that Cx43 and Cx37 are modulators of leukocyte trafficking into inflamed tissues$^{12-14}$ and are implicated in atherosclerosis.$^{10,11}$ We have generated an atherosclerosis-susceptible mouse line in which Cx40 is specifically

![Figure 7. Regulation of CD73 activity and leukocyte adhesion by Cx40-mediated GJIC. A through D, Cx40 immunostaining (green) in confluent cultures of bEnd.3 cells incubated with Cx40-sense, SCx40 (A) or Cx40-antisense, ASCx40 (B) oligonucleotides or transfected with lipofectamine (C) or lipofectamine+Cx40 siRNA, siCx40 (D). Cells were counterstained with Evans blue dye (red) and nuclei with DAPI (blue). Bar=32 μm for A and B and 50 μm for C and D. E, mRNA was isolated from untreated and siCx40-treated bEnd.3 cells and subjected to reverse transcription polymerase chain reaction with the use of specific primer pairs for Cx40 (lanes 1 to 3) or GAPDH (lanes 4 and 5). The amplification product for Cx40 relative to GAPDH was markedly decreased in bEnds.3 cells treated with Cx40 siRNA (lanes 2 and 5) compared with controls (lanes 1 and 4). Lane 3: no reverse transcription. F, Western blot for CD73 in bEnd.3 cells incubated with SCx40 or ASCx40. Cont indicates control. G, Decreased Lucifer yellow diffusion in confluent cultures of bEnd.3 cells incubated with ASCx40 or transfected with siCx40. n=17 to 25. *P<0.001. H, Transfection of bEnd.3 cells with siCx40, but not siCx26, decreased cell surface activity of CD73, as revealed by the malachite green assay. AMT indicates aminopterin, n=4 to 10. *P<0.01. I, Increased adhesion of mononuclear H36.12j cells on aminopterin-treated monolayers of bEnd.3 cells preincubated with ASCx40 compared with SCx40. n=8. *P<0.05. J, Increased adhesion of neutrophils on aminopterin-treated monolayers of bEnd.3 cells transfected with SCx40 compared with SiCx40. The effect of siCx40 on neutrophil adhesion was reversed by the A$_2$B receptor agonist NECA. n=4. *P<0.01.
Similar to mice with ubiquitous deletion of Cx40, the endothelial-specific deletion of Cx40 was associated with decreased expression of Cx37 in the aorta. The mechanism of this apparent coregulation of Cx37 and Cx40 is at present not known. Simon and McWhorter reported that ablation of Cx40 had greater effect on dye transfer between ECs than ablation of Cx37. In addition, we demonstrated, using in vivo adoptive transfer, that atherosclerotic lesion development in Cx37−/− ApoE−/− mice was caused by elimination of Cx37 in monocytes but not in the endothelium. It is thus not likely that decrease in Cx37 expression is solely responsible for the Cx40-knockout phenotype, but its contribution in inhibiting GJIC cannot be ruled out.

Under normal and pathological circumstances, circulating leukocytes migrate from vessels into tissues by a multistep process involving the sequential activation of adhesion proteins and their ligands on both leukocytes and ECs. A possible explanation for the early plaque development in Cx40del mice may be an intrinsic activation of ECs. In support of this hypothesis, we observed enhanced infiltration of macrophages in aortic sinuses as well as increased expression of VCAM-1 in aortic ECs of Cx40del mice compared with their controls. Because VCAM-1 has a dominant role in the initiation of atherosclerosis, endothelial Cx40 may play an antiadhesive role during leukocyte–EC interaction at early stages of the disease. This idea was strengthened by the observation that deletion of Cx40 in the lung capillary bed was associated with increased early recruitment of neutrophils to the inflamed alveolar space. Possibly, Cx40-mediated GJIC may regulate or synchronize the expression of adhesion molecules at the surface of ECs. In this context, we observed that CD73 expression was decreased in Cx40del aortic and lung capillary endothelium. This observation is consistent with the phenotype reported for CD73−/− mice, showing increased vascular expression of VCAM-1 and intercellular adhesion molecule-1 and exhibiting constitutive upregulation of the proinflammatory transcription factor nuclear factor-κB. Recent abstract publications reported enhanced inflammation and increased early atherosclerotic lesion development in CD73−/−/ApoE−/− mice. In agreement with our study, this supports the view that CD73-derived adenosine acts as an endogenous modulator protecting against chronic vascular inflammation and leukocyte recruitment. With respect to other vascular connexins (Cx43 and Cx37), the proatherogenic phenotype described here is unique to endothelial Cx40 deletion. Mice heterozygous for Cx43 (Cx43+/− LDLR+/−) showed decreased atherosclerosis, and enhanced plaque development in Cx37−/− ApoE−/− mice was ascribed to the absence of Cx37 in monocytes. Although deletion of the Cx40 gene may inhibit CD73 by various means, we hypothesize in this study that the lack of Cx40 may interrupt the spread of CD73-dependent signals between ECs during leukocyte–endothelium interaction.

CD73 is a membrane-bound glycoprotein that hydrolyzes extracellular adenosine nucleotides into bioactive nucleoside intermediates. Extracellular purines are released by various cell types, including leukocytes and ECs, and act as signaling molecules to mediate proinflammatory and antiinflammatory effects in vascular cells and leukocytes. For instance, the conversion of 5′-AMP into adenosine by endothelial CD73...
results in the activation of 4 transmembrane-spanning adenosine receptors. Among these receptors, the adenosine A2B receptor enhanced endothelial barrier function and hampered adhesion of leukocytes to the endothelium via a cAMP-dependent intracellular signaling. This cAMP-dependent signaling, in turn, further increases the expression and activity of CD73 and its antiadhering effect on leukocytes. We show here that Cx40 is not expressed by macrophages and neutrophils and thus cannot function as a hemichannel to transport nucleotides out of these leukocytes, as proposed recently for Cx37 and Cx43. Instead, specific targeting of Cx40 with antisense and siRNA reduced GJIC and CD73 activity and enhanced monocyte and neutrophil adhesion to ECs. Finally, the use of a coculture system showed that focal activation of A2B receptors conveys antiadhesion signals for leukocytes via functional Cx40 intercellular channels. Although the antiadhesion signals remain to be identified, cAMP appears as a good candidate because it is generated by the CD73 pathway. In addition, it permeates Cx40 channels, and elevation of intracellular cAMP or activation of A2B receptors upregulates GJIC in Cx40-transfected SKHeP1 cells, pituitary folliculostellate cells, and bEnd.3 cells (Figure 8A and 8B). Altogether, these observations indicate that Cx40-mediated GJIC regulates CD73 expression and activity, which in turn modulates the expression of adhesion molecules for leukocytes at the EC surface.

The beneficial role of Cx40 in the development of inflammation in various tissues has gained interest. Indeed, CD73-mediated adenosine production was identified in innate protective vascular inflammation, restenosis of wire-injured carotid arteries, acute lung injury, and cardiac allograft vasculopathy. We propose that the Cx40-dependent regulation of CD73 may contribute to the spatial expansion of antiinflammatory and antiadhesive responses within the endothelium. This mechanism, however, is impaired by deleting Cx40 in ECs, likely explaining the enhanced recruitment of leukocytes and the accelerated atherosclerosis observed in Cx40-deficient mice. Continued research efforts are performed to develop pharmaceutical strategies aimed at preventing or treating inflammatory diseases by controlling leukocyte recruitment to inflamed tissues and migration across the endothelium. Cx40 represents a novel target for the development of molecules that can enhance endothelial GJIC, which may be beneficial not only in atherosclerosis but also in other acute or chronic inflammatory diseases, as illustrated by the wide clinical use of the antiinflammatory molecule methotrexate.

Finally, polymorphisms in the promoter of the Cx40 gene have been associated with hypertension in humans. Because these polymorphisms likely affect the level of endothelial Cx40 expression, our data call for epidemiological studies focused on the possible use of these polymorphisms in cardiovascular disease risk assessment.

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The antiinflammatory mechanism of methotrexate depends on extracellular conversion of adenine nucleotides to adenosine by ecto-5'-nucleotidase: paracrine pathway for enhanced endothelial activation.


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Supplemental Methods

Generation of the $\text{Cx40}^{\text{fl/fl}}$ mice

A genomic clone derived from 129SV mice (9 kb HindIII-HindIII fragment, a kind gift of Dr. David Paul, Harvard Medical School, Boston, MA) and spanning the Cx40 exon 2 region was subcloned in pBluescript-KS. The gene-targeting construct was generated by replacing the HindIII-ScaI fragment, by the $\text{LoxP} - \text{neo}^r - \text{LoxP} - \text{Cx40 ORF} - \text{LoxP}$ cassette including Cx40 exon 2 upstream (Hind III-PmlI) and downstream (SnaI-SnaI) regions available for homologous recombination (Figure S1A). Transfection, selection (Figure S1B), and screening were performed as described by Verbeek et al. (1995) except that the selection medium contained 300 $\mu$g/ml neomycin and, in a subsequent selection round, 300 $\mu$g/ml G418. Clones bearing a targeted Cx40 allele were identified by Southern blot analysis of KpnI-digested genomic DNA using an exon 2-spanning probe. Hybridizing bands of 4 kb or 5.3 kb indicate the presence of Cx40 wild-type or Cx40 mutant alleles, respectively. From 900 neo$^r$ clones isolated, 17 (2%) had acquired the neo$^r$ insertion by homologous recombination. Of these clones, 4 did not show random integration; i.e. these 4 clones were killed by the G418 selection and were propagated from duplicate plates. Two clones were selected for microinjection of day 3.5 C57BL/6 embryos. Of these two clones, chromosomal counts were performed (Figure S1C) and found normal. Male chimeric offspring were bred with C57BL/6 females and the tail DNA of agouti offspring was analyzed for the presence of the mutated allele. Tail DNA from the offspring was analyzed by PCR for the presence of the mutated allele. The following primers were used: mCx40-46: 5’-

GTGACATGACCTGGATCTCTGGAG-3’ and mCx40-53: 5’-

GCCATCCTCTGCTACATATGCAG-3’; as well as the nested set mCx40-46a: 5’-
GTTAGAATCAATCCGACTCAC-3’ and mCx40-53a: 5’- GCAGCTAGAGCCATGAGTC , giving rise to a 555 bp (wild-type allele) and a 697 bp (mutated allele; including loxP) band. Heterozygous offspring carrying the mutation were backcrossed for two generations with Balb/c animals (Charles River, Iffa Credo, Lyon) and subsequently intercrossed. Homozygous Cx40<sup>fl/fl</sup> and wild-type littermates were selected to establish two independent Cx40<sup>fl/fl</sup> mouse strains with indistinguishable phenotype and the proper (Balb/c/Ola129) wild-type control strain. Then, Cx40<sup>fl/fl</sup> mice were backcrossed for 12 generations to the C57BL/6 background. Efficacy of in vivo recombination was first tested by crossing Cx40<sup>fl/fl</sup> mice with Cre-deleter mice. PCR was performed on genomic DNA isolated from tail-tips to evaluate excision of exon 2 of Cx40 and presence of Cre. Offspring of this crossing was heterozygous for the floxed Cx40 allele and half of the population expressed Cre. Figure S1D shows that mice that lack expression of Cre (lanes 3-5, 8 and 9) show the typical two banded pattern of wild-type (555 bp) and floxed Cx40 (697 bp). However, in mice expressing Cre (lanes 1, 2 and 6, 7) only the wild-type Cx40 band was detected while the higher band of 697 bp was absent, indicative of effective recombination.

**Atherosclerosis-susceptible mice with endothelial-specific deletion of Cx40 and EGFP mice**

For deletion of the Cx40 gene specifically in endothelium in vivo, we used a mouse strain expressing the Cre under control of Tie2 transcriptional elements. Endothelial specificity of recombination using this strain has been described previously. Thus, we interbred Cx40<sup>fl/fl</sup> mice with atherosclerosis-susceptible ApoE<sup>−/−</sup> mice to generate Cx40<sup>fl/fl</sup> ApoE<sup>−/−</sup> mice (control group 2; C2). Of note, wild-type mice do not develop advanced atherosclerosis. Interbreeding of ApoE<sup>−/−</sup> mice with mice harboring the Cre recombinase coding sequence under the control of the endothelial-specific Tie2 promoter resulted in Tie2Cre<sup>+</sup> ApoE<sup>−/−</sup> mice (control group 1;
C1). Finally, groups C1 and C2 were interbred to generate the Tie2Cre\textsuperscript{+} Cx40\textsuperscript{fl/fl} ApoE\textsuperscript{-/-} mice (Cx40del group). Genotypes of mice were checked by PCR using above described protocols.

Transgenic mice expressing ubiquitously the enhanced green fluorescent protein (EGFP) by a CMV-\(\beta\)-actin promoter or mice in which EGFP expression is under the control of the Cx40 gene (Cx40\textsuperscript{EGFP/+} mice) were also used.\textsuperscript{5,6} "Okabe" mice were kindly provided by Drs Hererra and Swetloff, Geneva University Medical School, Switzerland. All animal procedures were performed according to National Veterinary Guidelines and approved by the Ethics Committees of the individual Universities.

**Experimental interventions on mice**

Atherosclerosis. We induced atherosclerosis in control and Cx40del mice by feeding them from the age of 10 weeks with a high-cholesterol diet for 5 or 10 weeks. There were no significant differences in plasma lipids, leukocyte counts, and animal weights, between the three groups, both before and after the high-cholesterol diet (data not shown). Protocols for quantification of atherosclerosis have been previously described in detail.\textsuperscript{10}

Heart rates and arterial pressure measurements. All animals were anesthetized with isoflurane and implanted with microminiaturized radiotelemeters (PA-C20, Data Sciences Int., St Paul, MN) with catheter placed into the left carotid artery as described.\textsuperscript{8} Arterial pressure was recorded in conscious animals once daily for 5 d. at the same time starting from the day of operation. Heart rate was determined offline from the pressure curve.

Vasomotor responses in isolated aortas. Mice were anesthetized with pentobarbital sodium (40 mg/kg body weight, i.p.) and the descending thoracic aorta with intact endothelial layer was isolated and dissected free from surrounding tissue and cut into rings (3 mm in length).

The rings were suspended in a Multi-Myograph System (Model 610M, Danish Myo Technology A/S, Denmark) as described.\textsuperscript{9} Aortic rings were allowed to equilibrate for 45 min and progressively stretched to a passive tension of 2.5 mN which results in an optimal length–
tension relationship. The contractility was examined with the responses to KCl (100 mM) or NE (10^{-9} to 10^{-5} M); the endothelium-dependent and –independent relaxations were examined with the concentration response curves to ACh (10^{-9} to 10^{-5} M) or SNP (10^{-10} to 10^{-5} M). Relaxations were expressed as percentage of decrease in tension of the contraction to NE (3x10^{-7} M). Contractions were presented as percentage of tension compared to KCl.

Lung inflammation studies. Alveolar recruitment of neutrophils was measured in response to Pseudomonas aeruginosa LPS intra-tracheally (IT) instilled in anesthetized mice. Neutrophils were collected by BAL 3, 6, 12 or 24 h after LPS treatment. The collected cells were counted and stained with May-Grunwald-Giemsa for microscopic identification. In the absence of LPS, 90-95% of the cells were alveolar macrophages, the remaining being lymphocytes. In the presence of LPS, the proportion of neutrophils increased with time of stimulation to reach 80% of the alveolar cell population within 12 h.

Macrophage isolation. Mice were intraperitoneally injected with 1 ml thioglycollate 4% (Sigma). After 3–4 d, mice were killed and leukocytes were obtained by peritoneal washing using 5 mM EDTA in PBS. Peritoneal macrophages were then purified by magnetic cell sorting with CD11b microbeads and LS columns (Miltenyi Biotec) or by using a differential attachment procedure.

**Western blotting and immunohistochemistry.** Western blotting and immunostainings were performed as previously described\(^7,10\) using antibodies against Cx43 (BD Transduction), Cx40 (Chemicon or ADI), Cx37 (ADI), von Willebrand factor (Dako) or CD73 (Santa Cruz or Pharmingen). An anti β-actin (Pharmingen) was used as control for protein loading. Fluorescent cells were viewed on an inverted TMD-300 microscope (Nikon) equipped with a 40X phase 3 DM objective with a numerical aperture of 0.7 (Zeiss). Images were captured with a Visicam digital camera (Visitron Systems) connected to a personal computer running Metafluor 4.01 software (Universal Imaging).
Adhesion assays

Adhesion assays were carried out using monolayers of bEnd.3 cells and H36.12j cells, a mononuclear mouse cell line as previously described, or neutrophils collected by BALs from mice subjected to lung inflammation with 100 ng LPS for 24h, as described above. Leukocytes were labeled with 5-(and-6)-carboxyfluoresceine diacetate (Molecular probes), according to the manufacturer’s protocols. Labeled leukocytes were added onto a confluent monolayer of bEnd.3 cells grown on gelatin-coated plastic dishes for 30 or 90 min at 37°C. At the end of the adhesion period, dishes were carefully washed not to detach adherent cells and fixed with 4% paraformaldehyde. Adherent leukocytes were counted under a microscope equipped for fluorescence detection using a 20X objective. One hundred fields were considered and adherent leukocytes were expressed as number per field.

RNA silencing, RNA anti-sense and RT-PCR

bEnd.3 cell monolayers were transfected for 12h with small interfering RNA for Cx40, Cx26 or CD73 (all from Santa Cruz) using lipofectamine (Invitrogen) and allowed to recover in complete culture medium for an additional 12h. Transfection conditions were optimised using the Block-it™ Fluorescent Oligo (Invitrogen). In some experiments, bEnd.3 cells were pre-incubated for 24 h with 10µM of Cx40 sense (5’-GTA TCA TGC CAT CCC AG-3’) or anti-sense (5’-GAA GCT CCA ATC GCC CAT-3’) phosphorothioated oligonucleotides. RNA was extracted with TRIzol (Invitrogen) and further purified using Quick Prep Micro mRNA Purification Kit (Amersham). Reverse transcription and PCR were performed for Cx40 and GAPDH using the following primer pairs: Cx40 sense (5’-ATG GGT GAC TGG AGC TTC C-3’), Cx40 anti-sense (5’-CAC AAA GAT GAT CTG CAG TAC CC-3’); GAPDH sense (5’-TGG TAT CGT GGA AGG ACT CAT GAC-3’), GAPDH anti-sense (5’-ATG CCA GTG ACG TTC CCG TTC AGC-3’).
CD73 activity

To determine CD73 activity, bEnd.3 cells were washed in a phosphate free incubation medium and in the absence of nucleotide. The reaction was started by the addition of 1 mM AMP in a solution containing (in mM): 2 MgCl₂, 120 NaCl, 5 KCl, 10 glucose, 20 Heps (pH 7.4). After 40 min, the solution was collected and the reaction stopped by adding 5% (w/v) TCA and placed on ice. The release of inorganic orthophosphate (Pi) was detected by the malachite green method. The Pi released from cells incubated without nucleotide was subtracted from the total Pi released during the incubation. CD73 activity (nmol/40 min) is calculated from a standard curve generated using increasing concentration of KH₂PO₄ as a source of Pi. For each experiment, the CD73 inhibitor AMP-CP was included as an additional control condition to ensure that Pi was released from the AMP-dependent CD73 activity.

To evaluate nucleotides hydrolysis by native CD73 in lung microvessels, cryosections were fixed in 4% paraformaldehyde and preincubated for 45 min at room temperature in 0.25 mM sucrose, 2 mM CaCl₂ and 50 mM Tris-maleate, pH 7.4. Enzymatic assay was carried out at 37°C the same buffer complemented with 2 mM Pb(NO₃)₂, 5 mM MnCl₂, 3% dextran T-250 and 100 µM AMP as substrate. In control experiments, the substrate was omitted. The Pi released from nucleotide hydrolysis is captured by lead and visualized by precipitation with 1% (NH₄)₂S. Sections were then mounted with Eukitt (Kindler GmbH & CO) and photographed. Intensity of lead precipitation per lung alveolar septa surface was quantified using an algorithm created in Metamorph (Universal imaging).

Hela cell co-culture

Parental and Cx40-expressing Hela cells were kindly provided by Dr. Klaus Willecke (Bonn University, Germany). Either the wild-type (WT) or the Cx40-expressing (Cx40) cells were plated on the bottom of 5 µm-pores Transwell inserts (Costar) before Cx40-expressing cells
were seeded on the top of the insert. Cells were seeded at high density (200’000 cells) and cultured for 48h to allow the formation of cell-cell contacts within the pores, as previously reported.\textsuperscript{14} Cells in the bottom chamber were then exposed to NECA for 3 hours prior to addition of leukocytes (THP-1, a human monocytic cell line) onto Cx40-expressing cells in the top chamber. THP-1 cells were first labeled -(and-6)-carboxyfluoresceine diacetate and then allowed to adhere for 30 min. The number of adherent leukocytes was expressed as described above.
Supplemental Figures and Figure Legends

Figure S1: Generation of mice with a conditional mutation in the Cx40 gene
A: Schematic representation of the gene-targeting construct. B: Procedures used to select clones bearing the Cx40 allele at the appropriate integration site. C: Karyotyping on the selected clones revealed normal chromosome numbers. D: PCR analysis on mice heterozygous for the floxed Cx40 allele revealed two bands, representing wild-type Cx40 and floxed Cx40 (bottom panel), in mice not expressing the Cre recombinase (top panel). As expected, only the wild-type Cx40 gene product was detected in mice expressing Cre recombinase.

Figure S2: Endothelial-specific Cx40 deletion in ApoE−/− mice does not alter aortic function
A: Maximal contraction obtained with 100 mM KCl in aortic rings from control (C1 and C2, black and open bars) and Cx40del (hatched bar) mice. N=6 per group. B: Contraction of aortic rings from control (C1, C2, black and open squares) and Cx40del (triangles) mice in response to norepinephrine (NE). Values are expressed as percentage of KCl-induced maximal contraction. N=6 per group. C,D: Endothelium-dependent relaxations in response to acetylcholine (ACh) and endothelium-independent relaxations in response to the NO donor sodium nitroprusside (SNP) in aortic rings of control (C1 and C2, black and open squares) and Cx40del mice (triangles). N=6 per group. Responses for different concentrations were compared using analysis of variance for multiple comparisons (ANOVA with Bonferroni multiple comparison test). Contraction in response to KCl or NE was not significantly different (P=0.51) in aortic rings from controls and Cx40del mice. Likewise, endothelium-dependent (ACh) and -independent (SNP) relaxation were comparable between the three groups (P=0.82 and 0.89, respectively).
**Figure S3: Cx40del mice exhibit accelerated atherosclerosis**

Representative photographs (N=3-5) of aortic sinuses (A) and arches (B) from C1 (left), C2 (middle) and Cx40del (right) mice that had received 5 weeks high-cholesterol diet. Samples were stained with Sudan-IV (red) for lipids and counterstained with Mayer Hemalum (purple).

**Figure S4: Cx40 is not expressed by monocytes and neutrophils**

Fluorescent (A,B,D,E) and phase contrast (C,F) images of neutrophils (A-C) and monocytes (D-F) collected respectively from the alveolar space or peritoneal cavity of Okabe mice with systemic expression of EGFP or in mice in which EGFP expression is under the control of the Cx40 gene (Cx40<sup>EGFP/+</sup> mice). EGFP was not detected in both neutrophils (B,C) and monocytes (E,F) collected from Cx40<sup>EGFP/+</sup> mice. Bar represents 200 µm in panels A-C and 50 µM in panels D-F. G,H: Western blots for EGFP (top panels) and β-actin (bottom panels) in total proteins isolated from 1: parental Hela cells, 2: Hela cells transfected with a plasmid encoding for EGFP, 3: leukocytes from wild-type mice, 4: leukocytes from Cx40<sup>EGFP/+</sup> mice, 5: leukocytes from "Okabe" mice. Leukocytes are monocytes isolated from the peritoneal cavity in G and neutrophils collected from the alveolar space in H.
Supplemental References


Figure S1
Figure S2
Figure S3
Figure S4