

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.

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

Cardiovascular diseases currently constitute the major cause of death in developed countries.¹ Atherosclerosis, an inflammatory disease of large and medium-sized arteries,² is the most important cause of cardiovascular diseases. The main consequences of atherosclerosis are myocardial infarction, cerebral infarction, and aortic aneurysm.³

Clinical Perspective on p 9

Atherosclerosis involves the formation of intimal lesions that are characterized by a dysfunctional endothelium, inflammation, lipid accumulation, cell death, and fibrosis.^{2,3} The distribution of atherosclerotic plaques is highly charac-

teristic in humans; the lesions develop predominantly near side branches of arteries where blood flow is disturbed.⁴ 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.^{5,6} In this context, connexins, a large family of proteins that form hemichannels and gap junction channels enabling transmembrane and intercellular coordination of tissue activity,^{7,8} have been involved in atherogenesis. Three connexins are expressed in the vascular wall, namely connexin (Cx)37, Cx40, and Cx43, and important changes in their expression pattern have been reported in

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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.⁹ 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 immunostainings 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 H36.12j or THP-1 cells¹¹ 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 $\mu\text{mol/L}$ 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 nmol/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 α,β -methyleneadenosine 5'-diphosphate (AMP-CP) (50 $\mu\text{mol/L}$), the A₂B receptor antagonist alloxazine (10 $\mu\text{mol/L}$), or the A₂B receptor agonist 5'-(N-ethylcarboxamido)adenosine (NECA) (10 $\mu\text{mol/L}$) 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 \pm SEM. *P* < 0.05 was considered significant.

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 I 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 (*Tie2Cre⁺*) and atherosclerosis-susceptible apolipoprotein E (ApoE)-deficient (*-/-*) mice to generate *Tie2Cre⁺Cx40^{fl/fl}ApoE^{-/-}* mice (Cx40del) and control *Tie2Cre⁺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 aortic 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

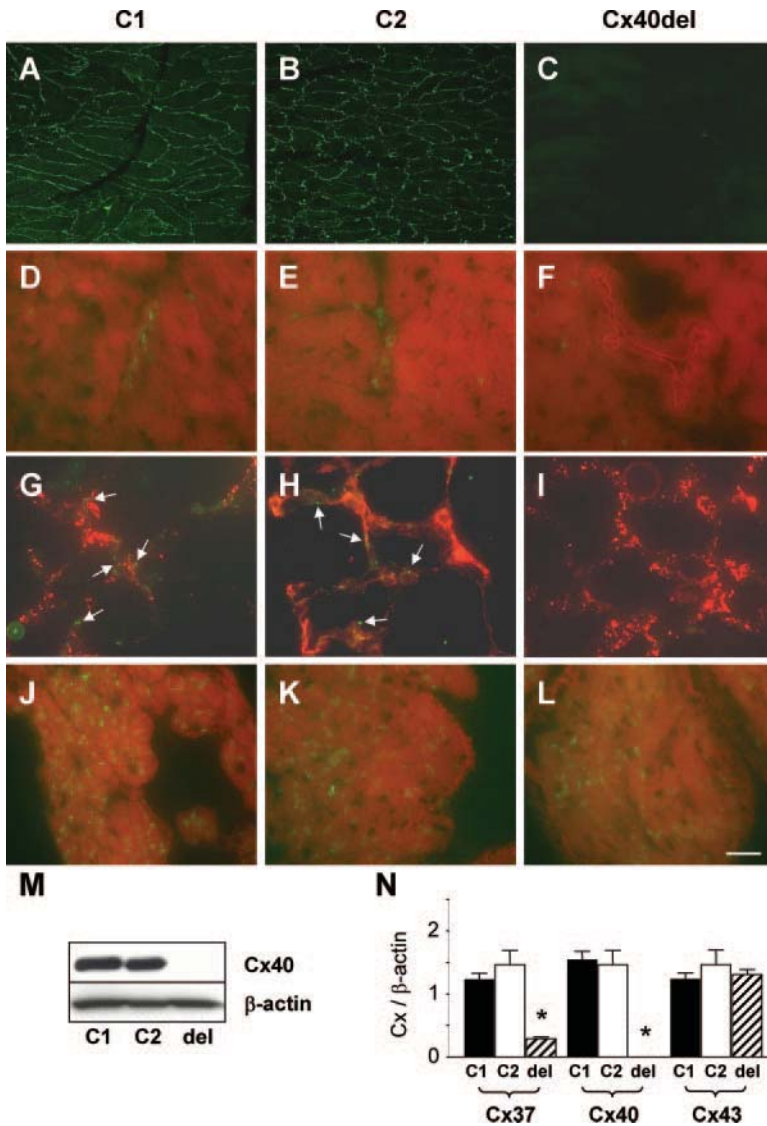


Figure 1. Endothelial-specific deletion of Cx40 in mice with the use of the Cre-LoxP system. A through I, Cx40 immunostaining (green) in thoracoabdominal aortas (*en face*, A to C) and cryosections of ventricular myocardium (D through F), alveolar septa (G through I), or atria (J through L) from *Tie2Cre⁺ApoE^{-/-}* (C1; A, D, G, J), *Cx40^{fl/fl}ApoE^{-/-}* (C2; B, E, H, K), and *Tie2Cre⁺Cx40^{fl/fl}ApoE^{-/-}* (Cx40del; C, F, I, L) mice. Heart sections were counterstained with Evans blue (red). Lung sections were immunostained with antibodies against von Willebrand factor (red) and Cx40 (green). Bar=25 μ m for A through C and G through I; 100 μ m for D through F and J through L. M, Representative Western blot for Cx40 in total protein obtained from thoracoabdominal aortas from C1, C2, and Cx40del mice. β -Actin was used as a control for protein loading. N, Bar graph showing quantification of Western blots for Cx37, Cx40, and Cx43 normalized to β -actin, from control (C1, C2) and Cx40del mice. n=4. * P <0.05.

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 (acetylcho-

line) 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 ($21.4 \pm 2.5\%$; n=10; P <0.01) compared with both C1 ($12.6 \pm 1.3\%$; n=10) and C2 ($7.1 \pm 1.3\%$; n=10) mice (Figure 3A). There was, however, no significant difference in Sudan IV staining in aortic sinuses from Cx40del ($29.1 \pm 6.7\%$; n=10), C1 ($34.9 \pm 7.5\%$; n=10), and C2 ($24.9 \pm 6.2\%$; 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

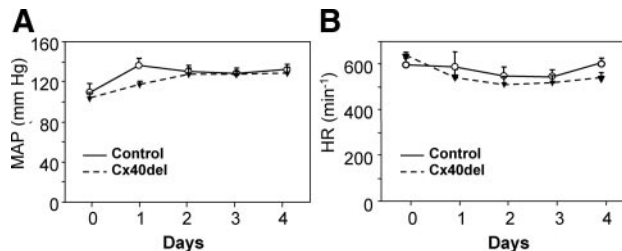


Figure 2. Cx40del mice are not hypertensive. A, B, Mean arterial pressure (MAP) and heart rate (HR) measured by telemetry in conscious control (circles; n=5) and Cx40del (triangles; n=8) mice demonstrate that Cx40del mice did not exhibit an elevated mean arterial pressure. An average for all days of mean arterial pressure and heart rate delivered P values of 0.22 and 0.23, respectively (unpaired t test).

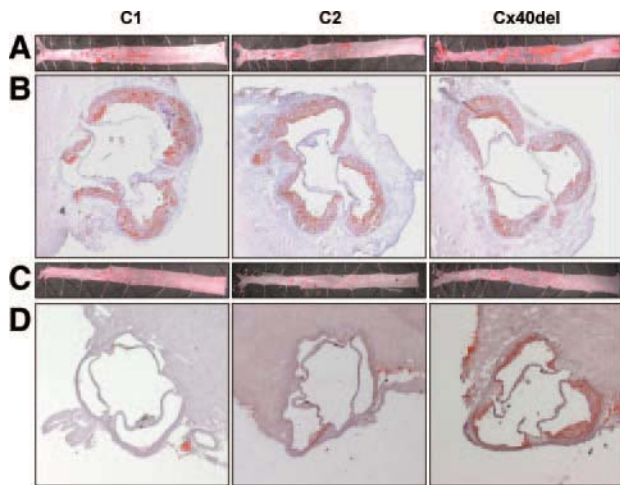


Figure 3. Cx40del mice exhibit accelerated atherosclerosis. Representative photographs (n=5 to 10) of thoracoabdominal aortas (A, C) and aortic sinuses (B, D) from C1 (left), C2 (middle), and Cx40del (right) mice that had received 10 weeks of a high-cholesterol diet (A, B), 5 weeks of a high-cholesterol diet (C), or did not receive a high-cholesterol diet (D). Samples were stained with Sudan IV (red) for lipids, and aortic sinuses were counterstained with Mayer hemalum (purple).

thoracoabdominal aortas of Cx40del mice ($5.2 \pm 0.6\%$; n=5; $P < 0.03$) compared with C1 ($2.9 \pm 0.6\%$; n=5) and C2 ($3.0 \pm 0.6\%$; 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, $21.4 \pm 8.3\%$; C1, $15.3 \pm 4.1\%$; C2, $12.2 \pm 3.8\%$; 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 ($0.5 \pm 0.1\%$; n=3) or C2 ($1.7 \pm 0.8\%$; n=3) mice. In contrast, Cx40del mice showed considerable ($P < 0.05$) lipid deposition at this location ($6.4 \pm 1.7\%$; 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 ($50.1 \pm 6.6\%$; n=5) after 5 weeks of high-cholesterol diet compared with C1 ($36.4 \pm 4.4\%$; n=5) and C2 ($41.0 \pm 5.4\%$; 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).

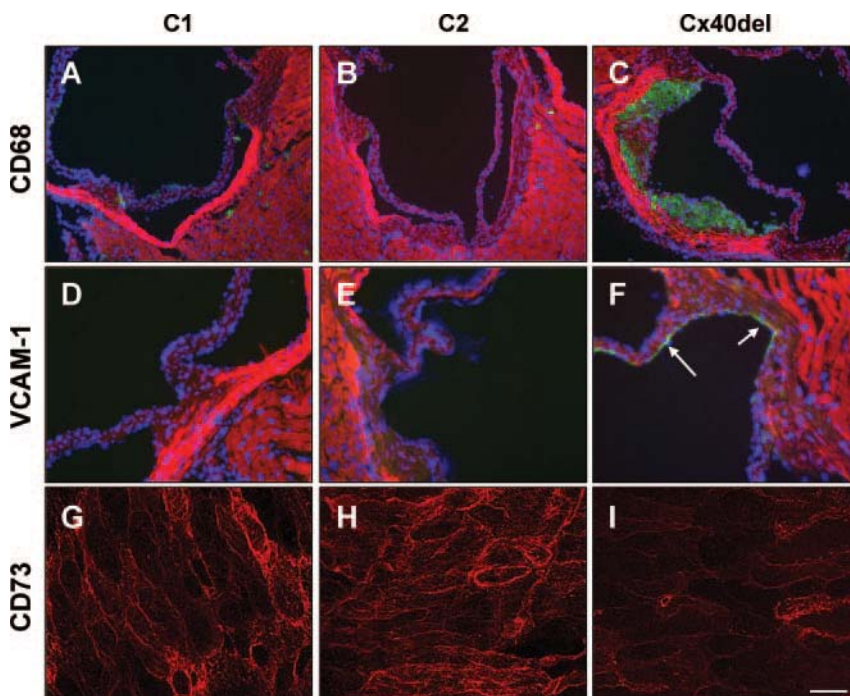


Figure 4. Cx40del mice show enhanced monocyte recruitment. Representative immunostainings (green) of cryosections from aortic sinuses of 10-week-old control (C1 and C2, left and middle, respectively) and Cx40del mice (right) for CD68 (A to C) and VCAM-1 (D through F). Sections were counterstained with Evans blue (red) and DAPI (blue). n=5. G to I, En face staining of thoracoabdominal aortas with a Texas red-conjugated antibody against CD73. Bar=200 μ m for A through C; 100 μ m for D through F; and 50 μ m for G through I.

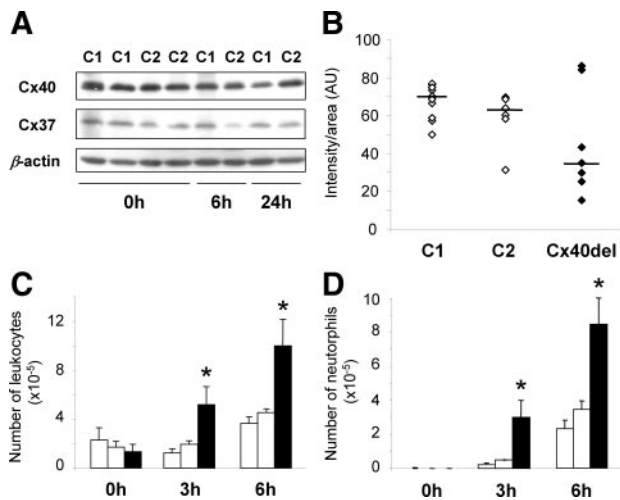


Figure 5. Cx40del mice showed enhanced recruitment of neutrophils to the alveolar space. A, Western blots for Cx37, Cx40, and β -actin from control mice. B, CD73 activity was determined by lead precipitation of inorganic orthophosphate release on lung cryosections. The intensity of the precipitate was calculated and compared between Cx40del and control (C1 and C2) mice. Bars represent medians. The total number of leukocytes (C) and of neutrophils (D) was determined in BALs from Cx40del (closed columns) as well as from control (open columns) mice after intratracheal instillation of lipopolysaccharide. $n=6$. $^*P<0.001$.

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 in control and Cx40del mice. 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 ($P<0.001$) at 3 and 6 hours after lipopolysaccharide treatment. 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,²³ 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 (*Cx40*^{EGFP/+} 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) and macrophages (Figure IVE and IVF in the online-only Data Supplement) from *Cx40*^{EGFP/+} mice. The

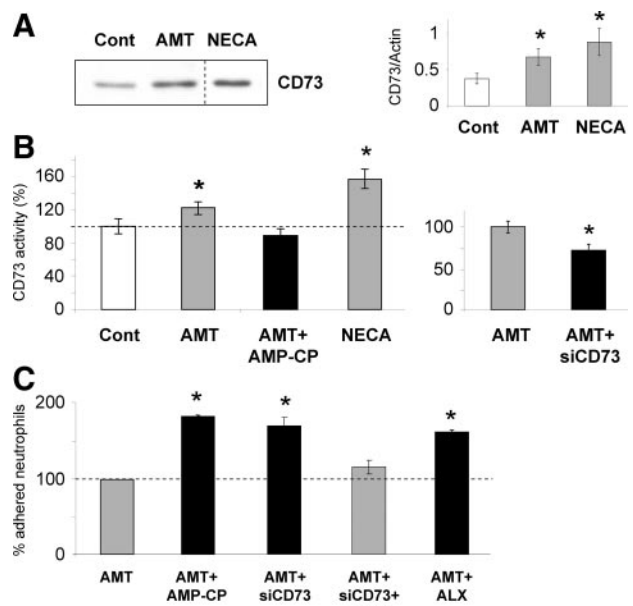


Figure 6. Inverse relationship between CD73 and leukocyte adhesion to ECs in vitro. A, Western blot for CD73 from bEnd.3 cells treated with aminopterin (AMT) or NECA. The dotted line indicates that the original gel was cut to illustrate the NECA condition close to the control (Cont) and aminopterin bands. Quantification after normalization of the CD73 signal to β -actin is shown on the right panel. $n=4$ to 11. B, CD73 cell surface activity, as revealed by the malachite green assay, on bEnd.3 cells treated with aminopterin, NECA, or aminopterin+AMP-CP. $n=17$ to 33 (left). Cell surface CD73 activity was also reduced after transfection of a specific siRNA for CD73 (siCD73). $n=4$ (right). C, Adhesion assay for mouse neutrophils onto bEnd.3 cells pretreated with aminopterin to increase functional CD73 expression. Blocking CD73 with AMP-CP, blocking A_2B receptors with alloxazine (ALX), or targeting CD73 with a specific siCD73 increased the adhesion of neutrophils to ECs. The latter effect was reversed by global activation of A_2B 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 A_2B receptors by adenosine, which results from the hydrolysis of adenine nucleotides by CD73.^{24,25} 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 4.²⁶ In contrast, the mouse bEnd.3 EC line constitutively expresses CD73 and Cx40 properly localized at cell-cell contacts.^{27,28} The methotrexate analogue aminopterin is known to promote adenine nucleotide release and thus the production of adenosine via CD73 activity.^{19,29} 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 A₂B 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 A₂B receptors with NECA (Figure 6C). Moreover, A₂B 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 cells¹¹ 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 A₂B 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 A₂B receptors enhanced GJIC, whereas blocking CD73 reduced 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

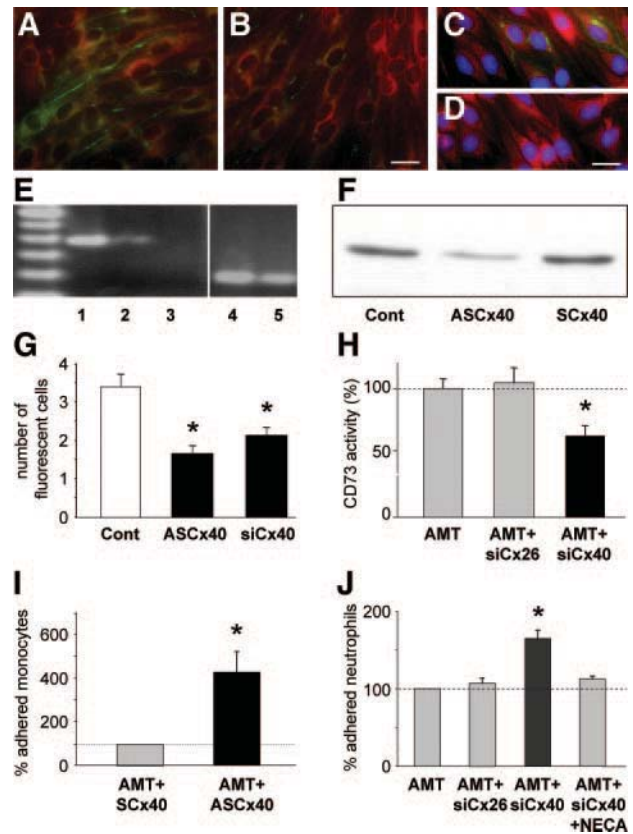


Figure 7. Regulation of CD73 activity and leukocyte adhesion by Cx40-mediated GJIC. A through D, Cx40 immunostaining (green) on confluent cultures of bEnd.3 cells incubated with Cx40-sense, SCx40 (A) or Cx40-antisense, ASCx40 (B) oligonucleotides or transfected with lipofectamine (C) or lipofectamine+ Cx40 siRNA, siCx40 (D). Cells were counterstained with Evans blue dye (red) and nuclei with DAPI (blue). Bar=32 μ m for A and B and 50 μ m 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 bEnd.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=6. * $P<0.05$. J, Increased adhesion of neutrophils on aminopterin-treated monolayers of bEnd.3 cells transfected with siCx40 compared with siCx26. The effect of siCx40 on neutrophil adhesion was reversed by the A₂B receptor agonist NECA. n=4. * $P<0.01$.

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

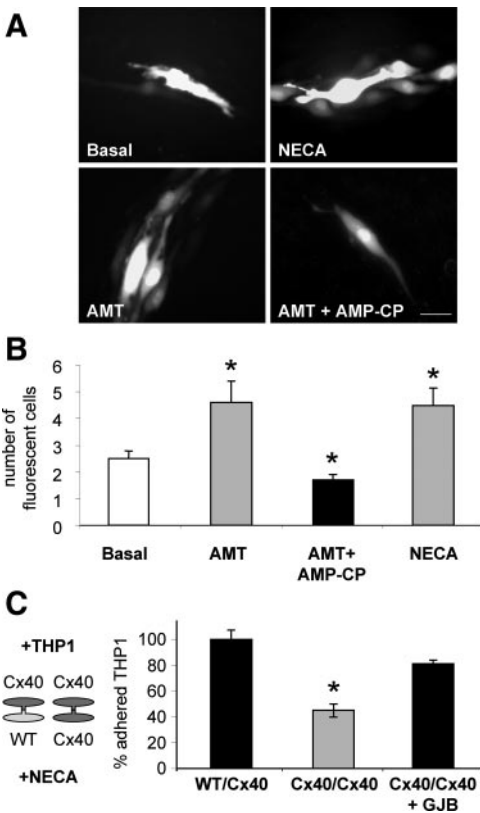


Figure 8. Cx40 intercellular channels convey antiadhesion signals for leukocytes. **A**, Representative dye injections of bEnd.3 cells exposed to NECA, aminopterin (AMT), or aminopterin+AMP-CP. Bar=50 μ m. **B**, Quantitative evaluation of the number of Lucifer yellow-labeled cells. $n=16$ to 20. * $P<0.001$. **C**, Cx40 intercellular channels are able to convey antiadhesion signals to unstimulated cells. In scheme, Cx40-expressing HeLa cells (dark gray) are on top of Transwell filters, and wild-type HeLa cells (light gray) or HeLa cells expressing Cx40 are on the bottom of the filters. The cells on the bottom are activated by NECA, whereas THP-1 leukocytes are plated onto Cx40-expressing cells on top of the filters. THP-1 cell adhesion was decreased in the Cx40/Cx40 cell combination compared with the WT/Cx40 combination, an effect that was reversed in the presence of 20 μ mol/L carbenoxolone, a gap junction blocker (GJB). $n=4$ to 5. * $P<0.001$.

deleted from ECs only. We demonstrate here that endothelial-specific deletion of Cx40 promotes atherosclerosis by increasing CD73-dependent leukocyte adhesion to the endothelium.

In contrast to mice with ubiquitous deletion of Cx40,^{17,18} mice with endothelial-specific deletion of Cx40 are not hypertensive. These results are in agreement with recent studies implicating Cx40 expression in renin-producing cells as crucial for blood pressure regulation.^{30,31} When exposed to an atherogenic diet, Cx40del mice and controls developed significant vascular lesions within 5 to 10 weeks. As expected, the atherosclerotic lesions are distributed near side branches of arteries, where blood flow is disturbed.^{4,32} Although atherosclerosis is known as a lipid-driven disease, shear stress has gained interest as an alternative or complementary explanation for plaque formation. Interestingly, aortic sinuses from 10-week-old Cx40del mice on a chow diet showed considerable atherosclerosis. Future studies on ApoE-expressing mice will reveal whether mice with endothelial-specific deletion of Cx40 develop atherosclerosis without the need for a cholesterol trigger.

Similar to mice with ubiquitous deletion of Cx40,²⁰ 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 McWhorter²⁰ 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.¹¹ 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.³³ 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,³⁴ 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- κ B.^{35,36} Recent abstract publications reported enhanced inflammation and increased early atherosclerotic lesion development in CD73^{-/-} ApoE^{-/-} mice.^{37,38} 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^{-/-}) show decreased atherogenesis, and enhanced plaque development in Cx37^{-/-} ApoE^{-/-} mice was ascribed to the absence of Cx37 in monocytes.^{10,11} 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 adenine 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.²⁴ 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.^{24,39,40} This cAMP-dependent signaling, in turn, further increases the expression and activity of CD73 and its antiadhering effect on leukocytes.²⁵ We show here that Cx40 is not expressed by macrophages and neutrophils and thus cannot function as hemichannels to transport nucleotides out of these leukocytes, as proposed recently for Cx37 and Cx43.^{11,41} 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,⁴² and elevation of intracellular cAMP or activation of A₂B receptors upregulates GJIC in Cx40-transfected SKHep1 cells,⁴³ pituitary folliculostellate cells,⁴⁴ 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 CD73 in the development of inflammation in various tissues has gained interest. Indeed, CD73-mediated adenosine production was identified in innate protection of vascular inflammation,^{25,35} restenosis of wire-injured carotid arteries,²² acute lung injury,^{45,46} and cardiac allograft vasculopathy.²⁷ 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 Cx40del mice. Continued research efforts are performed to develop pharmacological 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.⁴⁷ 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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Disclosures

None.

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CLINICAL PERSPECTIVE

Endothelial dysfunction, the initiating event of atherosclerosis, is characterized by increased expression of adhesion molecules and cytokines, which promotes the transmigration of leukocytes into the atherosclerotic lesion. This study provides evidence that connexin40 (Cx40), a gap junction protein expressed in endothelial cells, regulates the activity of the membrane-bound 5'-ecto-nucleotidase (CD73). The activity of endothelial CD73 generates adenosine from the hydrolysis of adenine nucleotides. Adenosine, in turn, activates surface membrane receptors to trigger antiadhesion signals for leukocytes to the endothelium. We have generated an atherosclerosis-susceptible mouse line in which Cx40 is specifically deleted from the endothelium. Endothelial deletion of Cx40 accelerated atherosclerotic lesion formation and coincided with increased expression of vascular cell adhesion molecule-1 as well as decreased expression of CD73. The antiadhesive role of Cx40 was confirmed in an endothelial cell line by specific targeting of Cx40 with antisense and small interfering RNA. We also found that functional Cx40 intercellular channels convey antiadhesion signals for leukocytes. Thus, Cx40-dependent regulation of CD73 may contribute to the spatial propagation of antiinflammatory and antiadhesive responses within the endothelium. These findings provide a molecular basis for therapeutic modulation of Cx40-mediated intercellular communication, which may be beneficial not only in atherosclerosis but for other inflammatory diseases as well. Of note, Cx40 gene polymorphisms affecting protein expression levels have been associated recently with hypertension in humans. Future investigations should determine whether these polymorphisms might be of use in atherosclerosis risk assessment.