Inactivation of CD73 promotes atherogenesis in apolipoprotein E-deficient mice

Anja Buchheiser, Annette Ebner, Sandra Burghoff, Zhaoping Ding, Michael Romio, Claudia Viethen, Antje Lindecke, Karl Köhrer, Jens W. Fischer, and Jürgen Schrader

1Department of Cardiovascular Physiology, Heinrich-Heine-Universität Düsseldorf, Universitätstr. 1, 40225 Düsseldorf, Germany; 2Biological and Medical Research Center (BMFZ), Heinrich-Heine-Universität Düsseldorf, Universitätstr. 1, 40225 Düsseldorf, Germany; and 3Institute of Pharmacology and Toxicology, Heinrich-Heine-Universität Düsseldorf, Universitätstr. 1, 40225 Düsseldorf, Germany

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Aims

CD73 (ecto-5′-nucleotidase) is expressed by a broad range of immune cells and attenuates inflammation in several acute disease models. This study therefore explored the role of CD73-derived adenosine in a model of chronic vascular inflammation such as atherogenesis.

Methods and results

CD73−/− mice were backcrossed into the apolipoprotein E (ApoE−/−) background. In CD73+/−/ApoE−/− double mutants, atherosclerotic lesion formation was increased by ~50% compared with ApoE−/−. However, the cellular composition and extracellular matrix of the plaques did not differ. Surprisingly, we found significant activity and expression of CD73 in the plaque of ApoE−/− mice which increased over time. CD73 co-localized with macrophages, Tregs, and cells of mesenchymal origin. Genome-wide microarray analysis of the aorta lacking CD73 revealed upregulation of endothelin-1 (Edn1) mRNA together with changes of genes in lipid metabolism and the Wnt and nuclear factor kappa B pathways. Measurement of plasma levels verified the upregulation of Edn1 in CD73−/− and double mutants. Plasma triglycerides (TG) were also found to be significantly elevated in the CD73−/−/ApoE−/− mice compared with ApoE−/− controls.

Conclusion

Lack of CD73 promotes atherogenesis most likely by de-inhibition of resident macrophages and T cells. Elevated Edn1 and TG levels may have contributed. This establishes CD73-derived adenosine as a direct or indirect regulator of atherogenesis.

Keywords

Adenosine • Plaque • Treg • Endothelin-1 • Triglycerides

1. Introduction

Ecto-5′-nucleotidase/CD73 is a 70 kDa GPI-anchored cell surface enzyme catalysing the extracellular conversion of nucleotide monophosphate esters into respective nucleosides.1 Dephosphorylation of 5′-AMP to adenosine, catalysed by ecto-5′-nucleotidase, is the final step of the extracellular nucleotide triphosphate breakdown cascade.2 Various physiological and pathophysiological events are modulated by interstitial adenosine via adenosine membrane receptors, of which four different types have been characterized: A1, A2A, A2B, and A3.3 Because the affinity of these receptors for adenosine differs, the adenosine signalling may largely depend on the interstitial adenosine concentration, which is importantly modulated by the activity of CD73.4

We have previously reported that the lack of CD73 in a mouse mutant is associated with a prothrombotic and proinflammatory phenotype.5 The enhanced adhesion of monocytes to the vessel wall under control conditions and after vessel injury was mediated by upregulation of nuclear factor kappa B (NFκB)-dependent adhesion molecules and was fully blocked by activation of adenosine A2A receptors.
2. Methods

The study was approved by the Institutional Animal Care and Use Committee of the Bezirksregierung Düsseldorf and conforms with the Guide for the Care and Use of Laboratory Animals published by the Directive 2010/63/EU of the European Parliament. All animals were bred at the central animal facility, Heinrich Heine University, Düsseldorf, Germany. For experiments, 6-month-old animals were killed by cervical dislocation before tissues were dissected.

2.1 Generation of CD73−/−/ApoE−/− mice

CD73−/− mice have been independently generated by three laboratories and have provided growing evidence that CD73-derived adenosine participates in numerous important biological functions, such as mediation of tubuloglomerular feedback, playing a crucial role in hypoxia-induced vascular leakage, being tissue protective in a model of bleomycin-induced lung injury, and enabling the efficient entry of lymphocytes into the central nervous system during autoimmune encephalitis.

It is becoming increasingly apparent that adenosine plays a central role in the regulation of the inflammatory response. Atherosclerosis is an inflammatory disease as well, characterized by intense immunological activity. Atherosclerotic plaques contain blood-borne inflammatory and immune cells, mainly macrophages and T cells aside from smooth muscle cells and extracellular matrix. Of particular interest is that macrophages and regulatory T cells (Treg) are able to degrade extracellular ATP to adenosine by action of CD39 and CD73. In this extracellular nucleotide cascade, ATP acts through various ATP receptors (P2-receptors), while its dephosphorylated end product, adenosine, acts through so-called P1-receptors. The functional significance of this pathway is that the activity of CD39 together with CD73 determines whether cells will be exposed primarily to a P1 or P2 environment. Lack of adenosine through action of various cytokines turns the local macrophages into a proinflammatory, activated phenotype. Similarly, when less adenosine is generated by CD73 on Treg, this will act on T effector cells to stimulate tissue inflammation and cell injury.

The present study explored the functional role of CD73 in the ApoE−/− background which have increased cholesterol plasma levels and extensive lipid deposition in major vessels such as the aorta, and the atherosclerotic lesions are infiltrated by CD4+ cells and macrophages. To this end, we created a CD73−/−/ApoE−/− double mutant and hypothesized that chronic lack of adenosine might exacerbate lesion formation. Furthermore, we aimed to explore some of the underlying cellular and molecular mechanisms.

2.2 Genotyping by PCR

Knockout of ApoE was tested by PCR (forward primer: 5′-CTCTACA CAGGATGCTTAGC-3′ and reverse primer: 5′-ATACTGTCCTCCTCAT CAGTGC-3′). Knockout of CD73 was tested with distinct primers for wild-type (forward primer 5′-CCCCTTTGGAGGTAAAGGAGA-3′ and reverse primer 5′-AGGAAAGGGTTCTCTTGA-3′) and knockout allele (forward 5′-TTGGGCTGCTCAAATAAG-3′; reverse 5′-CCT CAGTGTGAGTCTCA-3′).

2.3 Immunofluorescence

Immunofluorescence was performed on frozen tissue sections according to standard protocols with antibodies listed in the Appendix (see Supplementary material online).

2.4 Histology

Frozen sections (8 μm) of aortic sinus were stained according to Sirius Red or Smith Dietrich staining standard protocols. Affinity histochemistry of hyaluronic acid was performed as described previously.

2.5 Enzyme histochemistry

For localization of ecto-5′-activity, a lead phosphate precipitation method was applied to frozen tissue sections as described previously. To ensure that only CD73 and not alkaline phosphatase (AP) activity was detected, samples were blocked with 5% levamisole.

2.6 Quantification of aortic lesion area

Aortas were dissected from the aortic arch to the iliac bifurcation and briefly rinsed in phosphate-buffered saline. Peripheral adipose tissue and connective tissue were removed and the aorta was stained with Oil Red O according to the standard histological protocol. The stained aortas were opened longitudinal and fixed on agarose gel pads for photographing. Total and stained area were quantified by Photoshop 7.0 software.

For quantification of plaque size at the aortic root, frozen tissue sections were stained according to the Smith Dietrich staining. Plaque area was quantified with ImageJ.

2.7 Plasma and serum analysis

Commercially available ELISA kits were used for plasma analysis of macrophage chemotacttractant protein (MCP-1), interleukin (IL)-6, tumour necrosis factor-α (TNFα) (all from Endogen), and endothelin-1 (Edn1; R&D Systems). Fasting total, HDL, and LDL cholesterol were analysed by using the ChOD-PAP method (Boehringer, Mannheim). Triglycerides (TG) were analysed using Boehringer’s GPO-PAP method.

2.8 Microarray analysis

RNA was isolated using the RNAeasy Micro Kit (Qiagen). Amplification of RNA was accomplished by using an Amino Allyl MessageAmp Kit (Ambion). Thereafter, 5 μg of sRNA were labelled with Cy3 or Cy5 and hybridized on a genome-wide mouse OpArray™ (Operon). Three hybridizations were carried out in WT = Cy3/CD73−/− = Cy5 and three in WT = Cy5/CD73−/− = Cy3 orientation. Hybridization was performed in an HS 400 Pro hybridization station (Tecan). A GenePix 4000B laser scanner (Axon Inc.) was used for visualizing fluorescence signals and the GenePix Pro software (V 6.0) was used to calculate fluorescence intensities. The quality of each array hybridization was checked with the ‘mArray’ tool of the Biocosductor Package (http://biocosductor.org/). The background correction was done by the function ‘subtract’ and normalization was done with the standard parameters of the function ‘loess’. We defined all genes that met the following quality filter (flag count = 0 and signal to background ratio ≥ 3 in three of six arrays) and a P-value of <0.05 as significantly differentially expressed (see Supplementary material online, Table S1). P-values are based on calculations with the statistical analysis package Limma.

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2.9 Statistical methods

All results are presented as mean values ± standard deviation. Results were analysed by either two-way repeated-measures ANOVA as appropriate, followed by the Bonferroni post hoc test, or by Student’s t-test. All statistical analyses were performed using the SPSS software package. Differences were considered to be statistically significant at a value of P < 0.05.

3. Results

We have generated a mutant mice double-deficient for CD73 and ApoE by crossbreeding (CD73−/−/ApoE−/−). Mice were kept on normal chow diet, and at the age of 6 months, Oil Red O staining of aortae of wild-type, CD73−/−, ApoE−/−, and double-null mutants was performed to assess the extent of atherosclerosis (Figure 1). We found no plaque formation in the aortae of wild-type and CD73−/−− mice, but in ApoE−/− and ApoE−/−/CD73−/− double mutants. As shown, plaque formation occurred preferentially at loci of turbulent flow, such as ‘truncus brachiocephalicus’, ‘arcus aorta’, and some small branching of the ‘aorta descendence’. Plaque score was significantly increased in double-null mutants compared with ApoE−/− mice (5.5 ± 1.6% in ApoE−/− vs. 9.8 ± 4.5% in ApoE−/−/CD73−/−, P < 0.05, n = 8). Loci of atherosclerosis in double mutants were not limited to the small branching of the ‘aorta descendence’, but included almost all bifurcations. At the valvular cusp, plaque formation was with 33.2 ± 3.0% significantly (P = 0.044) higher in the ApoE−/−/CD73−/− mice compared with ApoE−/− controls (21.4 ± 3.5%, n = 5).

3.1 Histomorphology of plaques

A detailed histomorphological analysis of extracellular matrix composition in the plaques of aortic sinus was performed for ApoE−/− and ApoE−/−/CD73−/− mice, but not for wild-type and CD73−/− mice since no plaque formation occurred in these animals. The analysis revealed in both ApoE−/− and ApoE−/−/CD73−/− genotypes the establishment of collagen-rich plaques (Figure 2). The amount of collagen accumulation and the arrangement of collagen fibres were similar in ApoE−/−/CD73−/− double-deficient mice compared with ApoE−/− as indicated by Sirius Red staining viewed by light microscopy and by birefringence analysis, respectively. Also, the accumulation of non-collagenous extracellular matrix constituents such as hyaluronic acid was not affected in double-deficient animals. That the increase in plaque size at the aortic root was not paralleled by specific changes in extracellular matrix deposition was evaluated independently by three persons in a semi-quantitative manner. Furthermore, lipid retention as analysed by cholesterol staining was not different between the respective genotypes.

To evaluate the cellular composition of atherosclerotic lesions in ApoE−/− and ApoE−/−/CD73−/− animals, immunofluorescence analysis for the presence of monocytes/macrophages (CD11b) and T cells (CD4) was performed (Figure 3). We found high numbers of CD11b- and CD4-positive cells within the plaque area, but no difference between ApoE−/− and ApoE−/−/CD73−/− mice when the number of monocytes/macrophages and T cells normalized to plaque area were compared. In the ApoE−/− mice, 57 ± 15% of plaque cells were positive for CD11b, and in ApoE−/−/CD73−/− mice, the relevant number was 56 ± 8% (n = 5 each).

3.2 Expression of CD73 in plaques

In accordance with previous findings on the carotid artery, we found the expression and enzyme activity of CD73 in wild-type mice to be strongly associated with the endothelium of the aorta with some moderate expression/activity in the surrounding smooth muscle cells.

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Figure 1 Plaque formation in the aorta of WT, CD73−/−, ApoE−/−, and ApoE−/−/CD73−/− mice as visualized by Oil Red O in mice of 26 weeks. (A) Representative examples of each experimental group. (B) Quantification of plaque area in n = 8 animals in each group (*P < 0.05).
Unexpectedly, in ApoE\(2/2\) mice, there was a strong CD73 expression and activity at all small arteries branching off the aorta which co-localized with the atherosclerotic lesions (Figure 4A, middle). ApoE\(2/2\)/CD73\(2/2\) mice showed neither CD73 expression nor activity, proving specificity of the CD73 activity assay and the immunofluorescence antibody (Figure 4A, right). Similar results were obtained at sections of the aortic sinus, in which CD73 activity again co-localized with the plaque area (Figure 4B–F). CD73 activity increased with time and was strongest in 1-year-old ApoE\(2/2\) mice (Figure 4G). Higher magnification of the plaque area revealed that large round cells within the plaque (diameter up to 20 \(\mu\)m) were highly positive for CD73 activity (Figure 4E and F). Again, ApoE\(2/2\)/CD73\(2/2\) mice showed no CD73 activity (Figure 4D).

In an attempt to identify CD73-positive cells in the plaque of ApoE\(2/2\) mice, we analysed for cells known to possess CD73 activity such as foxP3-positive regulatory T cells, macrophages, and cells of mesenchymal origin (e.g. fibroblasts or smooth muscle cells). As shown in Figure 5, we found CD73-positive cells (red) preferentially within the plaque, but some also in the adventitial layers (Figure 5A, D, and G). Very few foxP3-positive cells (Treg; green) were found within...
the plaque area, but these cells did not co-stain for CD73 (Figure 5B and C). However, in the surrounding tissue, 53 ± 36% (n = 5) of the foxP3-positive cells were also positive for CD73 (yellow). Macrophages which are present within the plaque (Figure 5D–F) could be double-stained with anti-Galactin-3 (anti-Mac2; green) and anti-CD73 antibody (red). Quantification revealed that 38 ± 14% of all macrophages within the plaque area do express CD73 as well (Figure 5F). Fibroblasts are of mesenchymal origin and express vimentin. Therefore, double-immunofluorescence staining of cells within the plaque area against vimentin (green) and CD73 (red) was performed and revealed that 40 ± 5% of these cells stained positive for CD73 (Figure 5G–I). Another group of cells known to be present in plaques are smooth muscle cells and fibroblasts. Double staining of smooth muscle actin and CD73 revealed no co-localization of both proteins within the plaque area (data not shown).

3.3 Plasma cholesterol, MCP-1, TG, and free fatty acids

Serum total cholesterol levels were found to be elevated compared with wild-type controls in both groups examined (wild-type control: 85 ± 7 mg/dL; ApoE−/−: 318 ± 76 mg/dL; ApoE−/−/CD73−/−: 380 ± 44 mg/dL), but the differences between ApoE−/− and ApoE−/−/CD73−/− mice did not reach the level of significance. Similarly, LDL
cholesterol was elevated in both experimental groups to the same extent: wild-type control: 5 ± 1 mg/dL; ApoE<sup>−/−</sup>: 237 ± 55 mg/dL; ApoE<sup>−/−</sup>/CD73<sup>−/−</sup>: 245 ± 54 mg/dL. HDL cholesterol was comparable in all three groups (wild-type control: 72 ± 6 mg/dL; ApoE<sup>−/−</sup>: 102 ± 22 mg/dL; ApoE<sup>−/−</sup>/CD73<sup>−/−</sup>: 121 ± 19 mg/dL, n = 6 each).

Next, we explored whether there are differences in the plasma concentration of various cytokines, TG, and free fatty acids. MCP-1 plasma levels were elevated in both ApoE<sup>−/−</sup> and double-null mutant mice compared with wild-type or CD73<sup>−/−</sup> mice (Figure 6A). IL-6 and TNFα plasma levels, however, were comparable between all groups (see Supplementary material online, Figure S1). However, measurements of serum TG levels (n = 6 each) revealed significantly higher values in double-mutant mice (155 ± 22 mg/dL) compared with ApoE<sup>−/−</sup> control animals (97 ± 12 mg/dL, P = 0.027) (Figure 6B). We also measured the expression of adhesion molecules in ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>/CD73<sup>−/−</sup> mice. VCAM-1 was found to be present on smooth muscle cells located next to plaques, but no difference was observed between the two experimental groups (data not shown). Similarly, immunofluorescence analysis of the expression of other adhesion molecules like ICAM-1, ICAM-2, and E- and P-Selectin revealed no difference between ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>/CD73<sup>−/−</sup> mice (data not shown).
3.4 Microarray analysis of mouse aorta

In order to identify potential mechanisms underlying the observed CD73-dependent phenotype, we performed a genome-wide microarray study of the mouse aorta of CD73<sup>−/−</sup> vs. wild-type animals (<i>n</i> = 6). In this screening, we found 295 altered transcripts/genes, of which 231 are annotated (detailed data in Supplementary material online, Table S1). Genes significantly different between the two groups include genes regulating various signalling pathways such as Wnt (Wif1 (<i>P</i> = 0.0403, 1.25-fold), Tcf7l2 (<i>P</i> = 0.0154, 1.21-fold), Fzd4 (<i>P</i> = 0.0073, 1.28-fold), Jun (<i>P</i> = 0.0453, 1.20-fold), and Ccnd2 (<i>P</i> = 0.0073, 1.28-fold) and NFκB (Centb1 (<i>P</i> = 0.0333, 1.87-fold), Tlr2 (<i>P</i> = 0.0326, 1.53-fold), Traf2 (<i>P</i> = 0.0476, 1.20-fold), Tnfsf13 (<i>P</i> = 0.0363, 0.77-fold), Pla2g4a (<i>P</i> = 0.0333, 0.84-fold), Scd2 (<i>P</i> = 0.0021, 1.52-fold), and Vldlr (<i>P</i> = 0.0375, 1.21-fold). Since the observed expression differences were relatively small, we performed RT-qPCR analyses with several selected transcripts, to verify the microarray data. The results for Dnajb1 (1.88-fold by microarray, 1.33-fold by RT-qPCR), Edn1 (1.56-fold by microarray, 1.15-fold by RT-qPCR), Atf3 (1.55-fold by microarray, 1.61-fold by RT-qPCR), Pla2g4a (0.79-fold by microarray, 0.44-fold by RT-qPCR), and Ucp1 (0.73-fold by microarray, 0.75-fold by RT-qPCR) show the same regulation pattern as the microarray.

One of the genes most significantly upregulated in the microarray analysis was Edn1 (1.56-fold, <i>P</i> = 0.001), a known vasoconstrictor and modulator of atherosclerosis. To verify this microarray result again, we measured Edn1 expression in plasma of wild-type, CD73<sup>−/−</sup>, ApoE<sup>−/−</sup>, CD73<sup>−/−</sup>/ApoE<sup>−/−</sup>, and ApoE<sup>−/−</sup>/CD73<sup>−/−</sup> mutants. The data depicted in Figure 6C show that plasma levels of Edn1 in both CD73<sup>−/−</sup> and ApoE<sup>−/−</sup>/CD73<sup>−/−</sup>
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A critical initial step during the development of atherosclerosis is the adhesion of monocytes to the activated endothelium, followed by migration through the endothelium. In CD73−/− mice, the constitutive adhesion of monocytes in ex vivo-perfused carotid arteries was shown to be significantly increased and this proinflammatory effect was mediated by the upregulation of VCAM-1 demonstrated to occur at the protein and transcript level. In addition to the expression of adhesion molecules, the CC-chemokine ligand 2 (also known as MCP-1) produced by vascular cells is critical in guiding the recruitment of immune cells. In the present study, we found VCAM-1 to be unchanged in the plaque of double mutants while plasma MCP-1 levels were elevated which was dependent on the ApoE background. Gene array data of the aorta of CD73−/− mice in addition revealed upregulated toll-like receptor 2 (TLR2), which can elicit inflammatory responses directly. Thus, the vascular endothelium lacking CD73 is characterized by several factors synergizing in the recruitment of immune cells into the vascular lesion.

Immune cell function is also well known to be modulated by adenosine. When monocytes have finally differentiated into macrophages, they release inflammatory cytokines, chemokines, proteases, and radicals to cause tissue damage and maintain inflammation. Adenosine receptor activation strongly attenuates the production of several proinflammatory cytokines such as TNFα and IL-12, while at the same time, adenosine augments the formation of anti-inflammatory IL-10. Interestingly, in the present study, we found the expression and activity of CD73 is markedly upregulated in plaques of ApoE−/− mice. Immunohistochemistry revealed that 38% of the macrophages which are present within the plaque exhibit high CD73 expression. This may be interpreted as an attempt to limit the local inflammatory response. However, when extracellular adenosine is lacking, such as in CD73−/− mice, local macrophages turn into a proinflammatory, activated phenotype thereby enhancing atherogenesis. One therefore may speculate that overexpression of CD73 should be beneficial against atherogenesis.

In the present study, we found most of the foxP3-positive cells in the adventitia. Double staining with CD73 revealed that 53% of the foxP3-positive cells were also positive for CD73. This is of interest, as the expression and activity of CD73 is markedly upregulated in plaques of ApoE−/− mice. Immunochemistry revealed that 38% of the macrophages which are present within the plaque exhibit high CD73 expression. This may be interpreted as an attempt to limit the local inflammatory response. However, when extracellular adenosine is lacking, such as in CD73−/− mice, local macrophages turn into a proinflammatory, activated phenotype thereby enhancing atherogenesis. One therefore may speculate that overexpression of CD73 should be beneficial against atherogenesis.

In the present study, we found that lack of CD73 significantly accelerated the progression of atherosclerosis. It defines the extracellularly formed adenosine as an additional regulator built into the immune network to act as a protective factor in atherosclerosis which appears to be clinically relevant. The mechanism is complex and is likely to involve activation of vascular endothelium as well as activation of monocytes and T cells within the plaque when adenosine is lacking. Aside from a direct effect of adenosine, there appear to be indirect effects which at present are not fully understood resulting in the enhanced production of proatherosclerotic Edn1 as well as elevated plasma TG. Thus, CD73-derived adenosine appears to be an important extracellular metabolite modulating in vivo quite a variety of cellular functions which together determine the extent of plaque formation.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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