Regulation of CD73 in the development of lower limb atherosclerosis

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Abstract Atherosclerosis is an inflammatory process of the arterial wall. CD73 (also known as ecto-5'-nucleotidase) is a key regulator of cell signaling in response to inflammation and hypoxia, and may be important in the development of atherosclerosis. Recently, we have shown that high CD73 activity can be detected in the serum of patients with peripheral arterial disease (PAD). Using this same PAD patient cohort of 226 subjects with 38 femoral artery samples obtained during surgical endarterectomy and control artery samples taken during autopsy, we explored the association of serum CD73 activity with overall atherosclerotic burden and the expression of CD73 in mature and developing plaques. Interestingly, we found that CD73 activity had a tendency to increase along with more severe presentation of PAD (from 249 nmol/mL/h in moderate disease to 332 nmol/mL/h in severe disease; $P = 0.013$) and that CD73 expression is elevated in the vasa vasorum of developing plaques, but completely lost in mature occlusive plaques removed during endarterectomy ($P < 0.001$). The current findings implicate that as a result of shedding and loss of CD73 from the arterial wall, CD73 activity is elevated in the serum of patients with widespread atherosclerosis. These findings highlight the importance of a better understanding of the local role of CD73 in the development and maturation of arterial atherosclerotic plaques in man.

Keywords CD73 · Atherosclerosis · Purinergic signaling · Peripheral artery disease · Vasa vasorum

Abbreviations

PAD	Peripheral artery disease
TASC II	The second Trans-Atlantic Inter-Society Consensus for the management of peripheral artery disease
P-	Plasma alkaline phosphatase
AFOS	

Introduction

Purinergic signaling has become increasingly important in understanding vascular health and disease [1]. Impaired CD73-derived (ecto-5'-nucleotidase) adenosine production has been shown to contribute to the development of atherosclerosis both in mice and men [2–4] and also leading to the calcification of lower limb arteries in man [5]. Therefore, we recently explored CD73 activity in blood samples of a well-established cohort of patients with symptomatic lower limb atherosclerosis, which is more commonly referred to as peripheral artery disease (PAD) [6]. Against our expectations, we found that CD73 activity was significantly higher in blood samples of PAD patients when compared to age-matched healthy controls [7]. This did not support our original hypothesis that CD73 activity is impaired during the development of PAD and left us with two possible explanations; increased activity of CD73 in blood samples of PAD patients is a sign of increased activity of CD73 at the vascular wall or a sign of shedding and loss of CD73 in the vascular wall. Previous publications suggests the latter since significant elevation of circulating nucleotides ATP and ADP together with CD73 has been observed under hypoxic conditions [7, 8].

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To clarify this discrepancy, we correlated the extent of macroscopic presentation of lower limb atherosclerosis with serum CD73 activity and analyzed CD73 expression on mature occlusive plaques, developing plaques, and healthy lower limb vasculature.

Materials and methods

Study subjects and artery specimens

As a part of the PURE ASO Study (*The Role of Purinergic Signaling in Atherosclerosis*, approved by the local Ethical Committee of the Hospital District of Southwestern Finland), blood samples were gathered for 1 year from consecutive nonurgent elective PAD patients admitted to the Department of Vascular Surgery of Turku University Hospital, Finland [7]. During enrollment, 227 suitable patients were identified and 226 gave written informed consent. Out of these 226 PAD patients, 38 went through femoral artery endarterectomy in which the ruptured intima and thickened media occluding the artery were removed, leaving only the adventitia in place. CD73 expression analyses were performed from the shoulder region of the retained plaque. The shoulder region is the area where the plaque ends and turns into macroscopically normal vascular wall, i.e., it is the site where the endarterectomy and sample ends.

Under the institute approval of the Department of Pathology of Turku University Hospital, Finland, circular artery samples of macroscopically healthy femoral artery were taken from six unidentifiable subjects at autopsy without recording medical history. Only age at autopsy was recorded. These artery samples encompassed all layers of the arterial wall (intima, media, and adventitia).

Blood sampling and measurement of soluble CD73 activity in human serum

All samples were drawn in the morning prior to surgical procedures after at least 8 h of fasting. From all participants, 9 mL of whole blood was drawn to a serum sample tube and 9 mL to an EDTA sample tube. For EDTA plasma samples, the accredited University Hospital Central Laboratory conducted standard creatinine, lipid, glucose, and alkaline phosphatase (P-AFOS) analyses according to a photometric International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) procedure (values expressed as international units per liter, U/L). The serum sample tube was left to clot and cool down to room temperature for 3 h, while it was transported to the MediCity Research Laboratory, University of Turku. On arrival, it was centrifuged at 2000g for 10 min, after which the serum was extracted and stored at -70°C until analyses. CD73 activity was assayed by incubating 10 μL of

serum for 60 min at 37°C with 300 μM [$2\text{-}^3\text{H}$]AMP (Quotient Bioresearch, GE Healthcare, Rushden, UK). Radiolabelled substrates and their dephosphorylated products were separated by thin-layer chromatography and quantified by scintillation β -counting. Enzymatic activities were expressed as nanomoles of ^3H -substrate metabolized per hour by 1 mL of serum [9].

Immunohistochemical staining of CD73 expression in artery specimens

After obtaining the artery samples from endarterectomy or autopsy, the samples were directly moved to and stored at -70°C . Five micromoles thick frozen sections were fixed with acetone for 5 min and double stained with monoclonal antihuman CD73 (4G4) and biotinylated antihuman CD31 (eBioscience) antibodies or negative class matched control antibodies (all 10 $\mu\text{g}/\text{mL}$) by a two-step protocol where anti-CD73 was applied first for 1 h followed by incubations with an AP-conjugated antimouse IgG second stage reagent (1:100, 30 min in 5 % AB serum), APAAP reagent (1:50, 30 min in 5 % AB serum), and BCIP/NBT + levamisole (10 min) (all from DAKO) with $2\times$ PBS washes in between each incubation. After this, biotinylated antihuman CD31 was incubated for 1 h followed by a 30-min incubation with ABC reagent (Vectastain kit; DAKO) and a 5-min color development using AEC ready-to-use solution (DAKO) with $2\times$ PBS washes in between each incubation. The slides were mounted with Aquamount (Merck). Hematoxylin and eosin staining was performed on separate slides using ready-made solutions from DAKO. Quantification of CD73 staining intensity (0 no staining, 1 low staining, 2 moderate staining, 3 high staining) in CD31 positive structures was done from the arterial lumen and vaso vasorum of the vessel. Two independent observers did the quantifications.

Scoring the extent of atherosclerotic burden

The scoring of the extent of atherosclerosis of the lower limbs was done by two independent vascular surgeons using angiographic imaging, i.e., computed tomography angiography (CTA) or conventional digital subtraction angiography (DSA). Subjects with PAD were classified on a YES/NO basis on having a significant atherosclerotic lesion or occlusion in the following anatomical regions: aortoiliac, femoropopliteal, or crural arteries. A clinically significant lesion in only one anatomical segment gave an atherosclerotic burden score of 1 = moderate burden. Significant lesions in a combination of two anatomical regions, e.g., aortoiliaca and femoropopliteal arteries or crural and femoropopliteal arteries, gave a burden score of 2 = high burden. And, significant lesion in all three anatomical regions gave a burden score of 3 = severe burden.

Also, the TASC II score for the aortoiliac and femoropopliteal regions was used to estimate the effect of atherosclerotic burden on circulating CD73 activity. The TASC II classification grades severity and length of atherosclerotic lesions in four grades from A to D. TASC II A represents minor short clinically significant lesions and TASC II D represents long total occlusion [6]. We converted the A to D scoring into numbers as follows: 0 = no lesion in anatomical region, 1 = TASC A, 2 = TASC B, 3 = TASC C, and 4 = TASC D. Thus, the anatomical regions of aortoiliac and femoropopliteal arteries could separately get a score from 0 to 4, and these two scores were added together for a total TASC II score ranging from 0 to 8, which was then converted to similar 3° of TASC II-based atherosclerotic burden: moderate (TASC II combination score 1–3), high (TASC II combination score 4–6), and severe (TASC II combination score 7–8).

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows version 22.0. (IBM Corp. Armonk, NY, USA). Comorbidities were gathered on a yes/no basis and are presented as a percentage of prevalence amongst PAD patients. Differences in prevalence of baseline variables were tested using the chi-square test and the one-way ANOVA for numeric variables. Due to skewness, log-transformed CD73 activity values in serum were used to analyze the differences of CD73 activity with the extent of atherosclerotic burden. The Shapiro-Wilk test was used to ensure normality. The linear trend across the groups representing atherosclerotic burden (moderate, high or severe burden, or the sum of TASC II scoring) was then analyzed using linear regression fixed and nonfixed for plasma alkaline phosphatase (P-AFOS) values—a known colinear variable with circulating CD73 activity [7]. This was done to minimize the effect of individual variability of P-AFOS and the effect of underlying nonatherosclerotic conditions altering P-AFOS levels. CD73 staining between all healthy vessel samples and atherosclerotic plaques was tested using the independent sample *t* test and subject-specific variation of CD73 between a healthy vessel segment, and atherosclerotic segment was tested using the Mann-Whitney *U* test.

Results

Description of the study subjects

The PAD patient cohort used in this study has been presented previously in an exhaustive manner in relation to purinergic

signaling [7]. Shortly, the patient cohort consisted of 226 participants of which 128 (56.6 %) were male. All were of Caucasian origin. Overall, mean age was 69.8 years (SD $\pm$ 11.44, 46–93 years). In this context, the study group baseline data are presented in Table 1 in association with the degree of observed atherosclerotic burden based on the number of anatomical segments affected. The atherosclerotic burden groups were well matched and were without any significant differences according to age, gender, or cardiovascular risk factors (Table 1). In the group of severe atherosclerotic burden, there was a male dominance, slight increase in age, more smoking, and lower ABI implicating a more severe disease burden, but none of these measures showed a statistically significant difference.

CD73 activity in circulation increases along with the total atherosclerotic burden

In general, the measured CD73 activity was relatively high in patients with PAD when compared to known normal values and age matched healthy controls, as reported recently [7]. In addition, some exceptionally high values were measured (mean 253; SD 228; min 75, max 2568 nmol/mL/h). CD73 activity increased steadily in association with increasing atherosclerotic burden both as a measure of the affected anatomical segments (Fig. 1a) and as a measure of disease burden using the TASC II classification (Fig. 1b). Using log-transformed CD73 values adjusted for P-AFOS values and a linear regression model across atherosclerotic burden groups, CD73 activity increased identically no matter which disease burden measure was used (β = 0.16; SE 0.06; P = 0.013; for atherosclerotic burden by localization, and β = 0.15; SE 0.06; P = 0.014; for atherosclerotic burden by TASC II classification), see Figs. 1a, b. The corresponding measures regarding the association of atherosclerotic burden and CD73 activity without adjusting for P-AFOS values using a simple one-way ANOVA test for linearity were R = 0.15 (P = 0.025) and R = 0.14 (P = 0.043), respectively.

CD73 is lost from mature plaques

CD73 expression analyses revealed that plaque samples obtained from PAD patients during endarterectomy were consistently devoid of CD73. In general, these samples were very fibrotic and low in cellularity, which can be seen in the low nuclei count (Fig. 2a–c). Figure 2a, b illustrates the common scene from mature plaques of PAD patients. On the upper right hand corner of Fig. 2b, a small staining area for intact endothelium can be seen, but this is only positive for CD31 (endothelium positive control) and not for CD73.

Table 1 Baseline characteristics across atherosclerotic burden

	Moderate	High	Severe	<i>P</i> value ^a
<i>N</i>	65	117	44	
Male/female	52 %/48 %	54 %/46 %	70 %/30 %	NS
Age	69 (SD, 12.1)	72 (SD, 11.0)	73 (SD, 8.3)	NS ^b
History of smoking	55 %	55 %	73 %	NS
Hypertension	69 %	74 %	80 %	NS
Dyslipidemia	31 %	29 %	40 %	NS
Diabetes	28 %	39 %	36 %	NS
CKD	22 %	26 %	23 %	NS
Rheumatic disease	12 %	19 %	14 %	NS
ABI	0.49 (SD, 0.26)	0.46 (SD, 0.26)	0.39 (SD, 0.24)	NS ^b
P-AFOS	65 (SD, 22)	68 (SD, 31)	65 (SD, 34)	NS ^b
CD73 (nmol/mL/h)	249 (SD, 193)	269 (SD, 168)	332 (SD, 370)	<0.05 ^b
Medication				
Statin	63 %	62 %	68 %	NS
Aspirin	55 %	59 %	59 %	NS
Beta-blockers	51 %	57 %	63 %	NS
ACEi	45 %	44 %	46 %	NS
CCBs	26 %	33 %	32 %	NS
Furosemide	19 %	33 %	30 %	NS
Warfarin	23 %	18 %	29 %	NS
Nitroglycerin	20 %	29 %	21 %	NS
Metformin	19 %	18 %	18 %	NS
Cortisone	15 %	21 %	18 %	NS
ARBs	23 %	15 %	18 %	NS
Gliptins	9 %	13 %	11 %	NS
DMARDs	11 %	11 %	7 %	NS
Clopidogrel	6 %	8 %	16 %	NS
Bisphosphonates	6 %	8 %	5 %	NS

CKD chronic kidney disease, ABI ankle-brachial index, BP blood pressure, ACEi angiotensin converting enzyme inhibitors, CCBs calcium channel blockers, ARBs angiotensin receptor blockers, DMARDs disease modifying antirheumatic drugs

^a Chi-square test for differences across groups

^b One-way ANOVA for linearity across groups

Although the control autopsy group did not have PAD diagnosis, atherosclerotic process is unavoidable without clinical symptoms in an aging population, and thus, we found atherosclerotic changes at different stages in our control

population. This allowed us to explore CD73 expression at different phases in this disease. The control autopsy sample in Fig. 2c of a mature femoral artery plaque of a 79-year-old subject shows low cellular density and a highly fibrotic

Fig. 1 Logarithm transformed CD73 activity as nmol/mL/h across increasing atherosclerotic burden as a measure of **a** the number of anatomical segments affected and **b** TASC II scoring. Statistical linear association measured using a linear regression model fixed for P-AFOS values ($\beta = 0.16$; SE 0.06; $P = 0.013$ for **a**, and $\beta = 0.15$; SE 0.06; $P = 0.014$ for **b**)

Fig. 2 Examples of mature plaques stained with hematoxylin and eosin (H&E, left panels), or double stained with CD31 (red) and CD73 (violet) (right panels) showing fibrotic tissue, low cellularity, and poor CD73 expression. Specimens acquired during endarterectomy from the femoral artery of **a** PAD patient ID 66, **b** PAD patient ID 117, and **c** control femoral artery specimen from autopsy of a 79-year-old subject. The *arrows* in **b** show the endothelial lumen layer and in **c** mature plaque formation

sample similar to the samples from endarterectomy. On the contrary, a sample of healthy femoral artery of a 43-year-old subject taken during autopsy illustrates good cellular composition and shape of the vascular wall. High CD73 and CD31 immunoreactivity is seen in the small vasa vasorum on the outer wall of the sample artery and in addition moderate CD73 activity can be seen on the luminal surface of the endothelium in high magnification (Fig. 3a). In comparison, in a developing femoral artery plaque of a 66-year-old autopsy subject, a decrease in cellularity, increase in fibrotic tissue, and most importantly dilated and impregnating vasa vasorum of the vascular wall with high CD73 expression, but low CD31 expression, can be seen. The actual lumen side of the artery with slightly displaced endothelium is CD73-negative (Fig. 3b).

Quantification of CD73 staining was tested using the *t* test between all healthy autopsy samples and six plaque samples pooling both staining on the arterial lumen and vasa vasorum (Fig. 4a). CD73 staining was clearly higher in healthy vessels compared to mature atherosclerotic lesions ($P < 0.0001$). The same tendency was also seen in samples from the same individual. Autopsy control samples from the same individual

who had both atherosclerotic and healthy vessel segments showed that CD73 stained significantly less in the atherosclerotic lesion ($P = 0.029$) (Fig. 4b).

Discussion

Many scientific paths have led to the same conclusion that CD73 has a very profound regulatory role in inflammatory and hypoxic conditions [10–12]. Atherosclerosis is an inflammatory process of the arterial wall in which developing plaques cause thickening of the arterial wall further leading to local hypoxic conditions [13, 14]. Thus, it is logical to assume that CD73 would be an essential component of plaque development and maturation. Impairment of CD73 has been associated to the early development of PAD in the form of extensive calcification [5]. Recently, we studied the general level of CD73 activity in a cohort of patients with PAD but found no impairment of CD73. On the contrary, circulating CD73 activity was significantly higher in patients with PAD when compared to the age-matched controls [7].

Fig. 3 Examples of a healthy femoral artery (**a**) and slightly diseased femoral artery with a developing plaque (**b**) stained with hematoxylin and eosin (H&E, left panels), or double stained with CD31 (red) and CD73 (violet) (right panels). Specimen in **a** was acquired from autopsy of a 43-year-old subject showing intact and dense vascular structure with small and well staining vasa vasorum (indicated by arrows). A close up view of

the luminal surface endothelium staining for CD31 and CD73 is shown on the right hand side. Specimen in **b** was acquired from autopsy of a 66-year-old subject showing thickening of the arterial wall and dilated impregnating vasa vasorum (indicated by arrows) with overexpression of CD73. A foam cell cluster is circled by a dashed line in B. The lumen side is marked by an asterisk

According to the present findings, the high CD73 activity observed in the circulation of patients with PAD seems to be a result of shedding and loss of CD73 expression in mature occlusive plaques. This interpretation is supported by the association of increasing serum CD73 activity with the overall burden of atherosclerosis obtained using two different measures of disease severity. Comparably, Buccheiser et al. found that CD73 activity and expression in ApoE(−/−) mice

increases during the development of atherosclerotic plaques over time [3]. Unfortunately, in the current setting, this interpretation is limited by the fact that CD73 is an ubiquitous enzyme and circulating CD73 activity integrates shedding from various cellular sources and not only from endothelial cells. Circulating CD73 also arises from immune cells, particularly in inflammatory states, and from other organs. To minimize the effect that such subclinical conditions could have on

Fig. 4 CD73 expression on endothelial cells is significantly decreased in atherosclerotic plaques. **a** Healthy ($n = 8$) and diseased vessels ($n = 6$) were double stained for CD73 and CD31. Quantification of CD73 staining intensity (0 no staining, 1 low staining, 2 moderate staining, 3 high staining) in CD31 positive structures was done from the lumen (blue circles) and vaso vasorum (empty circles). Some diseased main vessels ($n = 3$) (negative for CD73) did not show CD31 immunoreactivity and were not included in the quantification. The quantification was done by

two independent observers. **b** Since some patient samples had both healthy vessel areas and plaque formation, we analyzed CD73 expression within close vicinity of these two different areas on the same vessel. Plaque included immune infiltration, foam cell formation, and thickening of the vessel wall and plaque. CD73 levels from healthy vaso vasorum (light green) and lumen (dark green) from tibialis, popliteal, and femoralis are shown as a reference

CD73 activity, we used plasma AFOS to adjust for this [7]. As we have previously shown, circulating CD73 activity has a positive correlation with P-AFOS values. Plasma AFOS can be elevated as a result of several subclinical conditions, which are not evident from patient charts, e.g., subclinical inflammation, clinically inevident liver or bone metabolism, etc.

CD73 expression is sensitive to hypoxia [15], which may reflect high CD73 immunoreactivity in developing plaques and arterial wall thickening as seen in Fig. 3b in which CD73 is highly expressed on the dilated vasa vasorum impregnating the vascular wall [16]. Vasa vasorum hyperplasia has been shown to be an early characteristic of plaque development, whereas macrophage accumulation and fibrosis are late-stage developmental characteristics [17]. This developmental spectrum can also be seen in our artery samples. Correspondingly, we have previously shown that a chronic hypoxic state leads to the loss of ectonucleotidase expression in the vasa vasorum and accumulation of nucleotides and increased vascular permeability [8], and that the loss of nucleotide converting enzymes (ecto-nucleotidases CD39 and CD73) leads to elevated levels of ATP and ADP exacerbating the formation of atherosclerosis and thrombosis [18]. This may well be the case in mature occlusive plaques and destruction of arterial wall configuration seen in the endarterectomy samples of this study.

Even though endarterectomy samples for analyses were taken from the shoulder region of the withdrawn sample, i.e., the region where the actual plaque ends and macroscopically normal artery continues, the samples were surprisingly fibrotic and low in cells. This phenomenon only became more prominent when moving to the inner regions of the endarterectomy sample, during which the samples became nearly impossible to dissect for staining. A similar observation was made by Mahmut et al. in a study of sclerotic aortic valves. They showed that CD73 was over-expressed in the developmental phase of aortic valve calcification compared to mature sclerotic valves. However, opposite to our findings, mature sclerotic valves obtained during valve replacement surgery still had a higher expression of CD73 when compared to completely healthy valves [19]. This of course does not directly correspond to the condition of mature ruptured occlusive arterial plaques.

We still cannot fully answer whether the elevated CD73 expression observed during the development of plaques and vasa vasorum hyperplasia is a compensatory mechanism against the ongoing inflammatory process or even a phenomenon leading to disease progression. Some reports suggest that increased CD73 expression, and accompanying increase in local adenosine levels could result in the progression of the sclerotic diseases via adenosine receptor signaling [19, 20]. In this respect, the individual differences in relation to adenosine receptor signaling in atherosclerotic disease progression remain a target for further studies. To this date, the

contemporary literature quite clearly highlights the anti-inflammatory role of CD73-mediated adenosine production [1, 12] and its importance for vascular integrity and beneficial remodeling [2, 21]. Thus, in accordance, we postulate that CD73 is elevated during the development of atherosclerosis to fight the disease and lost in the process of plaque maturity, plaque rupture, and adverse remodeling. In PAD, large segments of arteries are in a state of repair or disease progression. Based on the current observations, it seems reasonable to conclude that the increased CD73 activity in serum samples of PAD patients appears to be the result of shedding from the arterial wall because CD73 activity is absent in the plaques observed in severe PAD.

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Authors' contributions Initial idea and study design by JJ. HH and JJ collected the data. JJ, MH, and SJ contributed to the analysis of data. All authors were involved in preparing the manuscript and read and approved the final manuscript.

Compliance with ethical standards

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Ethical approval The study has been approved by the Ethical Committee of the Hospital District of South-West Finland and the study has been conducted according to the Declaration of Helsinki.

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