Role of the CD39/CD73 Purinergic Pathway in Modulating Arterial Thrombosis in Mice

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Objective—Circulating blood cells and endothelial cells express ectonucleoside triphosphate diphosphohydrolase-1 (CD39) and ecto-5'-nucleotidase (CD73). CD39 hydrolyzes extracellular ATP or ADP to AMP. CD73 hydrolyzes AMP to adenosine. The goal of this study was to examine the interplay between CD39 and CD73 cascade in arterial thrombosis. Approach and Results—To determine how CD73 activity influences in vivo thrombosis, the time to ferric chloride–induced arterial thrombosis was measured in CD73-null mice. In response to 5% FeCl₃, but not to 10% FeCl₃, there was a significant decrease in the time to thrombosis in CD73-null mice compared with wild-type mice. In mice overexpressing CD39, ablation of CD73 did not inhibit the prolongation in the time to thrombosis conveyed by CD39 overexpression. However, the CD73 inhibitor α-β-methylene-ADP nullified the prolongation in the time to thrombosis in human CD39 transgenic (hC39-Tg)/CD73-null mice. To determine whether hematopoietic-derived cells or endothelial cell CD39 activity regulates in vivo arterial thrombus, bone marrow transplant studies were conducted. FeCl₃-induced arterial thrombosis in chimeric mice revealed a significant prolongation in the time to thrombosis in hCD39-Tg reconstituted wild-type mice, but not on wild-type reconstituted hCD39-Tg mice. Monocyte depletion with clodronate-loaded liposomes normalized the time to thrombosis in hCD39-Tg mice compared with hCD39-Tg mice treated with control liposomes, demonstrating that increased CD39 expression on monocytes protects against thrombosis.

Conclusions—These data demonstrate that ablation of CD73 minimally effects in vivo thrombosis, but increased CD39 expression on hematopoietic-derived cells, especially monocytes, attenuates in vivo arterial thrombosis. (Arterioscler Thromb Vasc Biol. 2016;36:1809-1820. DOI: 10.1161/ATVBAHA.116.307374.)

Key Words: adenosine diphosphate ▪ monocytes ▪ nucleotidase ▪ platelet activation ▪ thrombosis

Acute myocardial infarction, secondary to platelet activation and arterial thrombus formation at the site of atherosclerotic coronary plaque rupture remains the leading cause of death in the Westernized World. In platelets, ATP and ADP are stored in the dense granules and released on activation. ATP and ADP interact with specific receptors on platelets (P2Y₁ and P2Y₁₂) to activate and recruit additional platelets to the site of vascular injury to form a thrombus. Within the vasculature, ATP and ADP levels are regulated by transmembrane nucleotidases that rapidly degrade ATP and ADP to diminish platelet activation and prevent thrombotic events. CD39 (ectonucleoside triphosphate diphosphohydrolase-1) is a 70 to 100 kDa transmembrane protein expressed on the surface of cells that circulate through the blood, lymph, and tissue and the surface of endothelial cells. CD39 hydrolyzes both ATP or ADP directly to AMP. AMP is then hydrolyzed to adenosine by CD73 (ecto-5'-nucleotidase). We have previously demonstrated that increased global expression of the human transgene CD39 (hC39-Tg) in mice conveys a profound antithrombotic effect. However, the cellular expression pattern necessary to provide this antithrombotic phenotype has not been elucidated. Although previous work suggests that endothelial cell–expressed CD39 and CD73 are key antithrombotic mediators and that hematopoietic expression of CD39 prolongs bleeding time in mice, the contribution of CD39 expression on hematopoietic-derived cells on thrombosis has not been elucidated in depth.

Given the critical role of purine nucleotide signaling in thrombosis and the expression of CD39 on hematopoietic-derived cells, we hypothesized that the level of CD39...
activity on bone marrow–derived cells could modulate in vivo occlusive arterial thrombosis. In this report, we demonstrate through bone marrow transplants (BMTs) and targeted depletion of monocytes, that increased CD39 expression on hematopoietic-derived cells, specifically monocytes, conveys resistance to in vivo thrombosis.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

Increased CD39 Expression Attenuates Platelet Activation

Collagen exposure after vascular injury initiates platelet activation. Whole-blood aggregation was conducted on blood from wild-type (WT) and hCD39-Tg mice to examine the effect of increased CD39 expression on platelet activation by collagen. In response to collagen stimulation of whole blood, platelet aggregation was significantly reduced in hCD39-Tg mice when compared with WT mice (Figure 1A through 1C).

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>APCP</td>
<td>α,β-methylene-ADP</td>
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<td>BMT</td>
<td>bone marrow transplant</td>
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<td>CD39</td>
<td>ectonucleoside triphosphate diphosphohydrolase-1</td>
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<tr>
<td>CD73</td>
<td>ecto-5′-nucleotidase</td>
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<tr>
<td>ENPP-1</td>
<td>ectonucleotide pyrophosphatase/phosphodiesterase 1</td>
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<tr>
<td>FACS</td>
<td>fluorescent activated cell sorting</td>
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<tr>
<td>FeCl₃</td>
<td>ferric chloride</td>
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<tr>
<td>GPₐβ₃</td>
<td>glycoprotein IIb/IIIa</td>
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<td>hCD39-Tg</td>
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**Figure 1.** Whole-blood aggregation, fibrinogen binding, and activated glycoprotein IIb/IIIa (GPₐβ₃)/P-selectin binding are decreased in human CD39 transgenic (hCD39-Tg) mice. **A**, Whole-blood aggregation curve in response to 1 mg/mL collagen in wild-type (WT) and hCD39-Tg mice. **B**, Aggregation and **C** Aggregation rate were measured at 6 min in WT (n=7) and hCD39-Tg (n=7) mice in response to 1 mg/mL collagen. **D**, Quantification of mean fluorescent intensity (MFI) from fluorescence-activated cell sorting (FACS) showing binding of anti-fibrinogen-fluorescein isothiocyanate-labeled antibody gated on platelets after challenge with 20µmol/L ADP from WT (n=3) and hCD39-Tg (n=3) mice. **E**, Representative dot plots (left) from FACS showing binding of P-selectin and activated GPₐβ₃ on platelets in response to thrombin-activating peptide 4 (TRAP-4) and percentage quantification (right) of platelets from WT (n=6) and hCD39-Tg mice (n=6). Bars in scatter plots represent mean±SEM. In (A) and (B), P values were determined with an unpaired t test with Welch’s correction. In (D) and (E), P values were determined using 1-way ANOVA with Tukey correction for multiple comparisons using the WT treatment group at each time point as a baseline comparison.
Similarly, fluorescent activated cell sorting (FACS) analysis showed that after ADP stimulation there was a statistically significant decrease in the binding of fibrinogen to hCD39-Tg platelets compared with WT platelets at 1 and 6 minutes (Figure 1D). Also, activation of platelets via thrombin-activating peptide 4 (TRAP-4) resulted in a statistically significant decrease in the percent of hCD39-Tg platelets expressing both activated fibrinogen receptor GPα IIb β3 and P-selectin when compared with WT platelets (Figure 1E). Further quantification of the mean fluorescent intensity (MFI) of both activated fibrinogen receptor GPα IIb β3 and P-selectin demonstrated a significant decrease in the MFI for both P-selectin and activated fibrinogen receptor GPα IIb β3 in hCD39-Tg platelets compared with WT platelets (Figure IA through ID in the online-only Data Supplement). Previous work has demonstrated that protease-activated receptor 4 activation is necessary for platelet thrombus propagation.17 Together, these data demonstrate that increased CD39 expression decreases the activation of platelets in response to collagen, ADP, and TRAP-4.

**Ferric Chloride Dose Effect With Ablation of CD73 and the Time to Arterial Thrombosis**

Although CD39 catalyzes the hydrolysis of ATP and ADP to AMP, AMP is hydrolyzed by ecto-5′-nucleotidase (CD73) to adenosine.18 Previous work has suggested that adenosine inhibits platelet activation.19,20 Also, previous work has reported that cd73-null (cd73−/−) mice display a reduced time to 7.5% FeCl3-induced carotid artery occlusion.21 To evaluate this mouse model, we first measured the expression of CD73 by FACS and showed that when compared with WT, Ly6C+ monocytes isolated from peripheral blood mononuclear cells are indeed null for CD73 expression (Figure 2A and 2B). To measure whether a more rapid time to occlusion occurred in cd73−/− mice, carotid artery injury was induced by a 5% FeCl3 solution, which resulted in a quicker time to thrombosis when compared with WT mice (Figure 2C). The average time to thrombosis was lower in cd73−/− mice compared with WT mice (WT: 16.8±2.03 minutes, n=12 versus cd73−/−: 11.87±1.16 minutes, n=13; P=0.05). However, in response to injury induced by 10% FeCl3, there was not a significant difference in the time to thrombosis between WT and cd73−/− mice (Figure 3E; WT: 12.69±2.17 minutes, n=6 versus cd73−/−: 9.7±1.64 minutes, n=5; P=0.31). These results suggest that the activity of CD73 can modulate the time to arterial thrombosis in response to less severe oxidative injury.

**Increased CD39 Expression Does Not Require CD73 Activity to Protect Against Arterial Thrombosis**

Our previous pharmacological studies suggested that CD73 activity is required for the antithrombotic phenotype observed in hCD39-Tg mice.11 Thus, our current finding that CD73 expression is not a critical regulator of arterial thrombosis against 10% FeCl3 conflicts with this previous work. Therefore, we examined whether CD73 activity is required for the antithrombotic effect in hCD39-Tg mice by using hCD39-Tg mice bred onto a cd73-null background (hCD39-Tg/cd73−/−). Representative FACS histograms (Figure 3A and 3B) and quantification (Figure 3C and 3D) show the concomitant knockout of CD73 and expression of hCD39 in hCD39-Tg/cd73−/− mice. Consistent with previous work,11 hCD39-Tg mice have a prolonged time to thrombosis (120±0.0* minutes; *an end-point of 120 minutes was used; >10× the duration of the time to thrombosis in WT mice) when compared with WT mice (12.6±2.17 minutes, n=5). Unlike previous data using 5% FeCl3 (Figure 2C), a 10% FeCl3 application did not change the time to thrombosis in cd73−/− mice (9.7±1.64 minutes, n=5) when compared with WT mice (Figure 3E). Surprisingly, in mice overexpressing hCD39, ablation of CD73 did not inhibit the prolongation in the time to thrombosis conveyed by hCD39 overexpression (hCD39-Tg/cd73−/−: 120±0.0* minutes; *an end-point of 120 minutes was used; Figure 3E). This finding seemed in conflict with our previously reported study demonstrating that the CD73 inhibitor αβ-methylene-ADP (APCP) nullified the prolongation in the time to thrombosis in hCD39-Tg mice.11 Therefore, we examined whether treatment of hCD39-Tg/cd73−/− mice with

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Ecto-5′-nucleotidase (CD73)-null mice have minimally increased thrombotic response to 5% ferric chloride (FeCl3). A through C, Eight-to-12-wk-old wild-type (WT) and cd73−/− mice were used for the following studies. A, Representative histogram gated on Ly6C+ peripheral blood mononuclear cells showing expression of CD73 and (B) a graph showing quantification of the mean fluorescent intensity (ΔMFI: antibody MFI – isotype control MFI) in WT (n=4) and cd73−/− mice (n=4). C, Time to thrombosis after 5% FeCl3 challenge in WT (n=12) and cd73−/− (n=13) mice. In (B) and (C), the P value was determined by unpaired t test with Welch’s correction. MFI indicates mean fluorescent intensity.
Figure 3. Resistance to arterial thrombosis does not require ecto-5′-nucleotidase (CD73) activity. A through C, Eight-to-12-wk-old wild-type (WT), human CD39 transgenic (hCD39-Tg), cd73−/−, and hCD39-Tg/cd73−/− mice were used for the following studies. A, Representative histogram gated on Ly6C+ peripheral blood mononuclear cells showing expression of hCD39. B, Quantification of the change in mean fluorescent intensity (ΔMFI: antibody MFI − isotype control MFI) of hCD39. C, Representative histogram gated on Ly6C+ peripheral blood mononuclear cells showing expression of CD73. D, Quantification of the change in mean fluorescent intensity (ΔMFI: antibody MFI − isotype control MFI) of CD73. E, Time to thrombosis after 10% ferric chloride challenge in WT (n=6), hCD39-Tg (n=4), cd73−/− (n=5), hCD39-Tg/cd73−/− (n=4), and hCD39-Tg/cd73−/− mice treated with 1 (n=7) or 2 (n=4) milligrams of αβ-methylene-ADP per kilogram of body weight. Time to thrombosis was considered to be 120 min. Bars in scatter plots represent the means±SEM. In B and D, the P value was determined by t test with Welch’s correction. In E, the P value was determined by 1-way ANOVA with Tukey correction for multiple comparisons. MFI indicates mean fluorescent intensity.
APCP affected the time to thrombosis. Treatment of hCD39-Tg/cd73\textsuperscript{−/−} mice with either 1 mg or 2 mg of APCP/kg of body weight resulted in abrogation of the thromboprotective phenotype conveyed by hCD39 expression (time to thrombosis: 1 mg APCP/kg body weight=10.4±3.33 minutes, n=7; 2 mg APCP/kg body weight=5.8±1.89 minutes, n=4; Figure 3E). These data suggest that at the APCP dosage used here and in previous studies,\textsuperscript{11,22} APCP may modulate arterial thrombosis through off-target effects. Indeed, previous work has demonstrated that in cell extracts, APCP can inhibit ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP-1), a related nucleotidase that hydrolyzes ATP or ADP to AMP.\textsuperscript{23} In this same study, APCP was found not to inhibit CD39 activity in cell lysates.\textsuperscript{23} However, we found that treatment of hCD39-expressing HEK cells with increasing concentrations of APCP demonstrated a decrease in total nucleotidase activity at a concentration of 10\textsuperscript{−4} to 1 mmol/L (Figure IIA and IIB in the online-only Data Supplement), suggesting that APCP may have off-target effects. Altogether, the genetic data demonstrate that CD73 expression is not required for the antithrombotic efficacy provided by increased CD39 expression in vivo and critically, that APCP has an off-target effect that negates the protection conveyed by hCD39 overexpression as evidenced in hCD39-Tg/cd73\textsuperscript{−/−} mice. With these key determinations, we next focused on understanding the cellular expression of CD39 that conveys resistance to thrombosis in vivo.

**Resistance to Arterial Thrombosis Is Transferred by Bone Marrow Transplantation**

Global overexpression of hCD39 provides a profound protection against arterial thrombosis\textsuperscript{11}; however, the cellular expression of hCD39 necessary for this vascular phenotype is not known. To examine the contribution of hCD39 expression on hematopoietic-derived cells or endothelial cells, BMTs were conducted between WT and hCD39 mice (Donor\textsuperscript{→}Recipient: WT\textsuperscript{→}WT, WT\textsuperscript{→}hCD39-Tg, hCD39-Tg\textsuperscript{→}WT, and hCD39-Tg\textsuperscript{→}hCD39-Tg). Sixty days after BMTs, complete blood counts (Table) and FACS analyses were performed on whole blood obtained from BMT recipient mice (Figure 4A and 4B). Comparable levels of leukocytes, red blood cells, and platelets were observed between the groups (Table). In addition to this, WT levels of endogenous mouse CD39 were not affected by BMT (Figure IIIA and IIIB in the online-only Data Supplement). FACS analysis confirmed functional BMT engraftment by showing hCD39-expressing bone marrow–derived cells in WT mice transplanted with hCD39 bone marrow and lack of hCD39 expression on bone marrow–derived cells in hCD39 mice transplanted with WT bone marrow (Figure 4B).

Cord arterial injury was induced by application of 10% FeCl\textsubscript{3}, and the time to thrombosis measured to examine the effect of increased CD39 expression on endothelial cells versus circulating blood cells in BMT recipient mice (Figure 4C). FeCl\textsubscript{3}-induced arterial thrombosis in chimeric mice revealed a significant prolongation in the time to thrombosis in recipient mice that received hCD39-Tg bone marrow (hCD39-Tg\textsuperscript{→}WT: 315.7±54.6 minutes and hCD39-Tg\textsuperscript{→}hCD39-Tg: 414.3±61.9 minutes). However, mice that received WT bone marrow did not have a prolonged time to thrombosis (WT\textsuperscript{→}WT: 7.97±0.6 minutes and WT\textsuperscript{→}hCD39: 17.15±6.0 minutes). Together, these data demonstrate that increased expression of CD39 on hematopoietic-derived cells rather than endothelial cells contributes to resistance to arterial thrombosis after vascular injury with 10% FeCl\textsubscript{3}.

**Increased Monocyte CD39 Expression Inhibits ADP-Induced Platelet Activation**

Given the vast distribution of CD39 expression on the vasculature and hematopoietic-derived cells,\textsuperscript{9,24} we examined the expression of hCD39, murine CD39, murine ENPP-1, and murine CD73 on endothelium and selected immune cell populations (Figures IV through VII in the online-only Data Supplement). Endothelial cells, T cells, B cells, and monocytes from hCD39-Tg mice all expressed increased levels of hCD39 (Figure IV in the online-only Data Supplement). No difference in endogenous mCD39 was observed between WT and hCD39-Tg endothelium or monocytes (Figure V in the online-only Data Supplement). ENPP-1 expression was low on both WT and hCD39-Tg endothelium and monocytes (Figure VI in the online-only Data Supplement). No difference in endogenous mCD73 was observed between WT and hCD39-Tg endothelium or monocytes (Figure VII in the online-only Data Supplement). No difference in endogenous mCD73 was observed between WT and hCD39-Tg endothelium or monocytes; CD73 was expressed on both. To begin to investigate which cell population contributes to the antithrombotic phenotype observed in hCD39-Tg mice, we initially focused on the monocyte population for several reasons. First, although endothelial cells express increased levels of CD39, the FeCl\textsubscript{3} model generates a strong oxidant injury;\textsuperscript{25} CD39 is inactivated by oxidant injury\textsuperscript{15}; therefore, we focused on the effect of CD39 on circulating cells. Given that under basal conditions >90% of circulating monocytes express constitutive CD39\textsuperscript{24} and because previous work has demonstrated that monocytes are one of the primary

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<th>hCD39-Tg</th>
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<td>hCD39-Tg</td>
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<td>n=5</td>
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<td>White blood cells, K/μL</td>
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<td>12.0±1.4</td>
<td>12.9±2.2</td>
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<tr>
<td>Red blood cells, K/μL</td>
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<td>9.5±1.0</td>
<td>9.6±0.2</td>
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<td>Platelets, K/μL</td>
<td>939.8±172.8</td>
<td>1180.0±180.3</td>
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\[ P \text{ value obtained with ANOVA test followed Tukey correction for multiple group comparisons. hCD39-Tg indicates human CD39 transgenic; and WT, wild type.} \]
cells recruited to sites of vessel injury, we examined the contribution of monocyte-expressed CD39.

We first asked whether increased numbers of WT monocytes, which express CD39, is sufficient to attenuate platelet activation. To answer this, we added increasing numbers of purified WT monocytes to diluted whole blood isolated from WT mice. Platelets were activated with 20 μmol/L ADP, and the expression of activated fibrinogen receptor GPαIIbβ3 was determined by FACS. There was no difference in WT platelet activation with the addition of purified WT monocytes (no monocytes: 1773±43.8 MFI, n=4 versus 104 monocytes: 1726±89.9 MFI, n=4, versus 105 monocytes: 1583±176.0 MFI, n=4; P>0.05; Figure 5A and 5B).

We then examined whether increased CD39 expression on monocytes attenuates platelet activation. To address this, we performed a similar experiment as outlined in the previous paragraph; however, instead of adding purified WT monocytes, we added purified hCD39-Tg monocytes. Representative histograms from FACS analysis (Figure 5C) and quantification of the MFI (Figure 5D) show increased activated GPαIIbβ3 integrin on WT platelets after stimulation with 20μmol/L ADP (1224±9.9 MFI, n=4). Additionally, mixing of 10^4 purified hCD39-Tg monocytes to WT whole blood produced a statistically significant decrease (663.5±61.5 MFI, n=4; P<0.001) in the exposure of the activated fibrinogen receptor, GPαIIbβ3 (Figure 5D). However, when a smaller number of purified hCD39-Tg monocytes, 10^4 cells, were added to WT blood (1160±92.3 MFI, n=4; P=0.97), there was no statistically significant difference in GPαIIbβ3. Altogether, these results show that increased expression of CD39 in circulating monocytes can decrease ADP-induced platelet activation.

Monocyte Depletion Abolishes the Antithrombotic Phenotypes of Increased CD39 Expression

Given that increased CD39 expression on monocytes reduced ADP-induced platelet activation ex vivo, we next examined whether increased expression of CD39 on monocytes modulates in vivo thrombus formation. We treated WT and hCD39-Tg mice with control liposomes and clodronate-loaded liposomes.
liposomes that deplete monocytes. Eighteen hours after clodronate liposome treatment, we observed a statistically significant and comparable reduction in the percentage of monocytes in both WT and hCD39-Tg mice (WT: control liposome: 53.2±3.3%; clodronate liposomes: 16.47±4.9%; P<0.001; hCD39-Tg: control liposome: 59.7±3.3% versus clodronate liposome: 22.3±2.3%; P<0.001; Figure 6A and 6B). After monocyte depletion, WT and hCD39-Tg mice were subjected to 10% FeCl3-induced carotid artery injury, and the time to arterial thrombosis measured (Figure 6C). Monocyte depletion reduced the time to thrombosis in hCD39 mice to that observed in both control and clodronate liposome–treated WT mice (hCD39-Tg: control liposome: 120.0±0.0* minutes; WT: control liposome: 8.62±1.3 minutes; clodronate liposome: 7.81±0.8 minutes). These results demonstrate that increased CD39 on monocytes can attenuate occlusive arterial thrombus formation in mice.

Discussion
In this study, we have demonstrated that increased CD39 expression on hematopoietic-derived cells, specifically monocytes, attenuates platelet activation and diminishes occlusive arterial thrombus formation in vivo. Furthermore, using genetic and pharmacological interrogation, we demonstrate that the activity of CD73 is not necessary for the
antithrombotic efficacy achieved with increased CD39 expression. Together, these data identify monocyte-expressed CD39 as a key regulator of platelet activation and arterial thrombosis.

Nucleotides released from injured or activated cells trigger purinergic receptor activation critical to thrombotic and inflammatory events. These nucleotide signals are distinguished by specific nucleotidases that hydrolyze nucleotides to nucleosides. The main pathway is the hydrolysis of ATP and ADP to AMP by CD39 followed by CD73-mediated hydrolysis of AMP to adenosine. CD73 is expressed on endothelial cells and some leukocytes.28–30 It has been reported that mice with a genetic ablation of CD73 have a decrease in the time to carotid arterial thrombosis after FeCl3-mediated vascular injury.21 Furthermore, it has been suggested that addition of a soluble form of CD73 to whole blood reverses ADP-induced platelet aggregation.31

In the current study, we demonstrated that ablation of CD73 activity had significant but temporally minimal effect on the average time to thrombosis in response to 5% FeCl3-induced carotid thrombosis (Figure 2E). However, in response to a more severe injury with 10% FeCl3, no difference in the time to thrombosis was observed in CD73-null mice. Our results obtained with 5% FeCl3 are consistent with data in the literature21; however, the effect of 10% FeCl3 was found not to be inhibited by the absence of CD73 in the present study. Therefore, we interpret the effect of CD73 on preventing arterial thrombosis as minimal. Most importantly, using genetic models, we found that ablation of CD73 activity did not abrogate the antithrombotic efficacy conveyed by increased expression of hCD39 in mice. This genetic finding was at odds with our previously published work demonstrating that treatment of hCD39-Tg mice with the CD73 antagonist APCP abrogated the protective phenotype observed in hCD39-Tg mice.31 Our observation that treatment of hCD39-Tg/cd73−/− mice with the CD73 inhibitor APCP abrogated protection conveyed by hCD39 overexpression, suggesting potential off-target effects of APCP in vivo. This finding contradicts a
recent study examining APCP analogs that reported no inhibition of CD39 activity by APCP when using cell lysates from hCD39-expressing cells.23 However, on the cell membrane, CD39 forms monomers, dimers, and tetramers that facilitate cooperative activity.32 Using HEK293 cells expressing human CD39, we observed that APCP treatment resulted in a decrease in CD39 activity in transfected cells (Figure II A and IIB in the online-only Data Supplement). Thus, APCP may affect yet unidentified pathways that regulate nucleotide metabolism and contribute to thrombosis. The fact that in mice lacking CD73 expression the CD73 inhibitor APCP abrogates the antithrombotic phenotype conveyed by overexpression of hCD39 demonstrates an off-target effect of APCP in vivo.

The generation of adenosine is limited in the vasculature to the activity of CD73 and circulating alkaline phosphatases. Previous work has suggested that CD73 and tissue nonspecific alkaline phosphatases comprise the major AMP-hydrolyzing enzymes in plasma.31 An alternate unexplored explanation is that APCP may inhibit tissue nonspecific alkaline phosphate activity. Thus, in the absence of CD73, as in our cd73−/− mice, tissue nonspecific alkaline phosphate metabolize CD39-generated AMP and thus still convey protection from thrombosis. However, with APCP treatment of cd73-null mice, the lack of both AMP-hydrolyzing pathways could result in an accumulation of AMP and end-product inhibition of CD39 activity. Alternatively, as mentioned earlier, APCP also inhibits ENPP-1, which metabolizes ATP or ADP to AMP. However, given the relatively low levels of ENPP-1 expression that we have demonstrated on endothelium and monocytes, the relative contribution of ENPP-1 to regulating thrombosis seems low.

Several studies have suggested that endothelial expressed CD39 is responsible for maintaining platelet quiescence under normal physiological conditions.8,15,34–37 However, with vascular injury, CD39 activity is lost.15,38 Furthermore, oxidative stress in endothelial cell injury can decrease CD39 activity.15,38,39 In humans, CD39 expression is lower in culprit lesions in patients with unstable angina compared with those with stable angina and is reduced in coronary atherectomy specimens with thrombus formation.40 Additionally, postmortem evaluation of patient’s samples deceased from acute myocardial infarction exhibit decreased CD39 enzymatic activity in tissue isolated from the coronary artery.41 Additionally, it has been reported that low levels of circulating CD39 activity are associated with critical limb ischemia in patients with peripheral atherosclerosis compared with patients with claudication.42 Therefore, CD39 activity or expression at sites of vascular injury or dysfunction has been postulated to be a critical regulator of thrombosis.

Previous studies have shown that increased CD39 expression can have wide regulatory effects in different pathological conditions. For example, increased CD39 expression on either vasculature or blood is sufficient to disrupt hemostasis and prolong bleeding time.12 Also, increased expression of CD39 can protect kidneys from ischemia–reperfusion injury43 and ameliorate streptozotocin-induced diabetes mellitus in mice.44 In addition to this, we have previously reported that hCD39-Tg mice have reduced platelet aggregation, prolonged bleeding times, and a marked resistance to arterial thrombosis.11 However, these previous studies did not elucidate the cellular requirement of increased CD39 expression to prolong the time to occlusive arterial thrombosis. Here, using complementary approaches of BMTs, ex vivo mixing studies and in vivo monocyte depletion studies, we have demonstrated that increased expression of CD39 on monocytes is capable of attenuating platelet activation and prolonging the time to occlusive arterial thrombosis. This finding is supported by a previous work suggesting that leukocyte-expressed nucleotidase activity may regulate platelet reactivity.45 First, the platelet aggregatory response to ADP is decreased in whole blood compared with that observed in platelet-rich plasma.45 Second, patients with leukocytosis have increased leukocyte CD39 expression and reduced ADP-induced platelet aggregation in whole blood.45,46 Last, the addition of polymorphonuclear and mononuclear leukocytes to platelet-rich plasma decreased ATP-induced aggregation.20 However, in this previous work, although it was suggested that nucleotidase activity on leukocytes was responsible for the antiaggregatory results, neither the specific nucleotidases nor the specific leukocyte populations responsible for this effect was identified. Together, these data suggested a role for leukocyte-expressed nucleotidases as a critical mediator in the cross talk that regulates platelet activation and aggregation. In the current study, we demonstrate for the first time that increased CD39 activity on monocytes can inhibit platelet aggregation and reduce the time to occlusive arterial thrombosis.

Previous work has shown that BMTs, where hCD39-Tg mice are used as donors, can lead to decreased levels of cellular reconstitution.47 This was evidenced by decreased percentage of donor hCD39-Tg cells in liver, spleen, and thymus after BMTs.47 In addition to this, hCD39-Tg mice show decreased percentages of MHC (major histocompatibility complex)–class II restricted CD4+ T cells and invariant natural killer T cells present in the liver and the spleen.47 Although CD4+ T cells48 and invariant natural killer T cells have been linked to atherosclerosis,49 the effect of these cell types in the acute setting of thrombosis has not been investigated. However, in the current study, we determined that after BMT, recipient mice transplanted with hCD39-Tg bone marrow had reconstituted CD45+ hCD39-expressing leukocytes (Figure 4A and 4B). The difference in cellular reconstitution could be because of the difference of BMT timing (42 days47 versus 60 days in the current study) or the fact that the current study focused solely on circulating blood cells rather than organs responsible for central/peripheral immunity.

Previous work has shown that CD39 activity can be retained in microparticles derived from platelets, endothelium, and leukocytes.50 The presence of microparticles has been shown in human plasma,51 and dysregulation of their activity has been associated with human disorders such as idiopathic pulmonary hypertension.52 Additional studies are needed to determine whether monocyte-derived microparticles modulate arterial thrombosis in the setting of increased CD39 expression. This avenue of research is of great interest because it would provide novel approaches for therapeutic intervention against arterial thrombosis.
In this study, we chose an updated model of the FeCl₃-induced model of arterial thrombosis that was initially developed in rats³¹ and later revised for mice.⁵ This mouse model has been reviewed extensively,²⁵,⁵⁵,⁵⁶ and relies on the application of FeCl₃ to the common carotid artery. FeCl₃-induced thrombosis has been used in numerous reports to study vascular occlusion in response to platelet surface molecules,²⁵,⁵⁸ P₂Y₁₂ receptor antagonism,²⁹ thrombin regulation,⁶⁰,⁶¹ cell cycle inhibition,⁶² factor Xa inhibition,⁶³ thrombolysis,⁶⁴ and multiple other pathways that can affect vascular function.⁶⁵–⁶⁷ We chose this model because FeCl₃-induced thrombosis is a model of thrombus propagation.²⁵,⁵⁵,⁵⁶ These mechanisms have been an essential tool for the understanding of multiple molecular pathways involved in thrombosis. However, this model is not without critique, and it is important to recognize that the exact mechanism by which FeCl₃ causes arterial damage is an important topic of debate.⁷¹ with recent work showing that the primary cell type binding damaged endothelial surfaces might be red blood cells rather than platelets.⁷² Regardless of the primary adhering cell, the FeCl₃-induced model of arterial thrombosis is a model of thrombus propagation.²⁵,⁵⁵,⁵⁶ These current explorations regarding the mechanisms involved in FeCl₃-induced arterial thrombosis not only allow us to make cautions interpretations about phenotypes observed in the FeCl₃-treated mouse but also highlight the importance of such a critical mouse model used to investigate human arterial thrombosis.

The relevance to human cardiovascular disease is that single-nucleotide polymorphisms rs10748643 and rs7071836 in the CD39 gene associated with the level of CD39 expression.⁷³,⁷⁴ We have demonstrated that the presence of rs10748643 genotypes associates with secondary cardiovascular events in patients with intracoronary stent placement for coronary artery disease.⁷⁵ Whether genetic determinants regulate CD39 expression on human monocytes is the focus of ongoing clinical studies.

In conclusion, increased CD39 expression conveys an antithrombotic phenotype that is independent of CD73 activity. Transfer of bone marrow with increased levels of CD39 is sufficient to transmit the antithrombotic phenotype of hCD39-Tg mice. In addition to this, depletion of monocytes ablates the antithrombotic effect of increased CD39 expression. Together, these data strongly suggest that increased CD39 expression on monocytes can attenuate platelet activation and in vivo thrombosis.

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Disclosures

None.

References


**Highlights**

- Increased expression of ectonucleoside triphosphate diphosphohydrolase-1 (CD39) in mice prevents platelet activation and arterial thrombosis.
- Eco-5′-nucleotidase (CD73) activity is not required to convey the antithrombotic phenotype transmitted by increased CD39 expression, suggesting that the contribution of CD73 in regulating thrombosis is minimal or can be compensated by alternate pathways.
- The CD73 inhibitor α-β-methylene-ADP blocks the protection from thrombosis in CD73-null/CD39-overexpressing mice suggesting off-target effects of α-β-methylene-ADP in vivo.
- Transplantation of hCD39-expressing bone marrow transmits thromboprotection.
- Increased expression of CD39 on monocytes prevents platelet activation and occlusive arterial thrombosis suggesting that modulation of CD39 activity on monocytes can prevent thrombosis.
Role of the CD39/CD73 Purinergic Pathway in Modulating Arterial Thrombosis in Mice
Roman Covarrubias, Elena Chepurko, Adam Reynolds, Zachary M. Huttinger, Ryan Huttinger, Katherine Stanfill, Debra G. Wheeler, Tatiana Novitskaya, Simon C. Robson, Karen M. Dwyer, Peter J. Cowan and Richard J. Gumina

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Materials and Methods

Mice
The generation of the human CD39 expressing (hCD39-Tg) mice has been described previously.1 The hCD39-Tg mice were backcrossed for more than ten generations onto the C57Bl/6 background and compared with wild-type (WT) littermate controls. The generation of CD73-null (cd73−/−) mice was described previously.2 The cd73−/− mice were obtained from Jackson Laboratory. The cd73−/− mice were backcrossed for more than ten generations onto the C57Bl/6 background and compared with WT littermate controls. The hCD39-Tg x cd73−/− mice were generated by crossing the cd73−/− mice and hCD38-Tg mice. Genotype was confirmed by polymerase chain reaction and fluorescent activated cell sorting (FACS) analysis. The investigations described conform to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by Vanderbilt University Institutional Animal Care and Use Committee and Ohio State University Institutional Animal Care and Use Committee.

Flow Cytometric Analysis and Platelet Activation
Platelets: FACS analysis was performed on whole blood obtained from the inferior vena cava of anesthetized WT or hCD39-Tg mice, and platelet activation was conducted as previously described.3 Briefly, platelets were activated by adding 20 μmol/L ADP and 1 mM CaCl2 and activation was measured by expression of activated glycoprotein IIb/IIIa (GPαIIbβ3; Clone: JON/A, Emfret), P-selectin (Clone: Wug.E9, Emfret) or binding of fibrinogen (rabbit anti-fibrinogen polyclonal IgG; P140-1, Emfret). The activation of platelets with protease-activated receptor 4 (Cat# 1487, Tocris Bioscience). Platelets were then analyzed in a 5-laser BD LSRII flow cytometer at the Vanderbilt Medical Center Flow Cytometry Shared Resource.

Blood monocytes: Whole blood was collected from the inferior vena cava of anesthetized WT, cd73−/−, hCD39-Tg or hCD39-Tg x cd73−/−. Blood was then incubated with Fc receptor block (BD Biosciences, Franklin Lakes, NJ) for 15 minutes at RT. Next, a cocktail of fluorochrome-conjugated antibodies (listed below) were added to blood samples and incubated for 30 minutes at RT. Following this, red blood cells (RBC) were lysed using BD Pharm Lyse (BD Biosciences) according to manufacturer’s protocol. After RBC lyses, cells were washed with FACS buffer (1X HBSS, 1% BSA, 4.1 mM Sodium bicarbonate, and 3 mM sodium azide) and the samples analyzed in 5-laser BD LSRII flow cytometer at the Vanderbilt Medical Center Flow Cytometry Shared Resource. The following fluorescent-labeled antibodies were used in this study: CD11b-Pacific Blue (Clone: M1/70, Biolegend, San Diego CA), CD45-FITC (Clone:30-F11, Biolegend), CD73-PerCP (Clone: TY/11.8, Biolegend), Ly6C-APC/Cy7 (Clone: HK1.4, Biolegend), hCD39-PE (Clone:498403, R&D Systems), mCD39 (Clone: 24DMS1, eBioscience) CD19-APC (Clone:6D5, Biolegend), TCRβ-V450 (Clone:H57-597, BD Biosciences) and CD31-PeCy7 (Clone: 390 BD Biosciences).

Mixing experiments
Whole blood was collected from the inferior vena cava of anesthetized WT or hCD39-Tg mice. Blood was processed with StemCell Technologies’ Easy Set Negative Selection Mouse Monocyte Enrichment Kit (Vancouver, Canada). Purity was confirmed routinely to be >80% by staining of monocytes (CD11b+, Ly6C+) by FACS as described above. Isolated monocytes in 10 μl volume from WT or hCD39-Tg mice were added to 25 μl of diluted whole blood at increasing cell numbers (0, 10⁴, 10⁵). Platelet activation was induced by stimulation with 20 μM ADP in the presence of 1 mM CaCl2. FACS quantification of activated GPαIIbβ3 was conducted as described above.
Whole blood aggregation
Blood was collected from the inferior vena cava of anesthetized WT or hCD39-Tg mice and whole blood aggregometry in response to collagen (1mg/ml) was analyzed as previously described.³

Bone Marrow Transplantation
Twenty-four hours before transplant, recipient mice received 100 cGy/minute for 5 minutes (RS-2000 Biological Irradiator, RAD-Source, Suwanee, GA) then 4 hours later the second dose of radiation for 4 minutes (100 cGy per minute) to ablate native bone marrow. Mice were placed on Baytril water treatment to prevent infection.

Donor mice were sacrificed under sedation (Ketamine: 80 mg/kg body weight, Xylazine: 15 mg/kg body weight). The femurs were then removed from the surrounding tissue. The epiphyses were removed and the bone marrow harvested by flushing the bones with RPMI media (CellGro, Manassas, VA). The bone marrow cells were passed through a filter to remove debris and then pelleted at 300 x g for 5 minutes. Red blood cells were lysed in lysis buffer (BioLegend, San Diego, CA) for 5 minutes, and remaining cells were pelleted by centrifugation at 300x g for 5 minutes at 4 degrees Celsius. The cell pellet was resuspended in RPMI with 5% (w/v) fetal bovine serum (FBS) to a final concentration of 50 x 10⁶ cells per milliliter of the buffer.

Bone marrow recipients received 5 x 10⁶ cells via tail vein injection approximately 24 hours after the last dose of radiation. Thrombosis studies were conducted 60 days after bone marrow transplant after confirming reconstitution.

Monocyte Depletion with Clodronate-Loaded Liposomes.
Monocyte depletion was conducted as previously described.⁴,⁵ Briefly, mice were anesthetized using ketamine (55 mg/kg) and xylazine (15 mg/kg) before intravenous administration of liposomal clodronate suspension or control liposome suspension (Encapsula Nano Sciences, Brentwood, TN). After 18 hours, 100 μL of whole blood were collected to assess the level of monocyte depletion.

In vivo carotid thrombosis
A 5% or 10% ferric chloride (FeCl₃) solution was used to induce carotid artery thrombosis as previously described.³ Because of the extreme prolongation in the time to the formation of an occlusive arterial thrombus in mice with increased expression of CD39,³ experiments were terminated at an end-point of 120 minutes after FeCl₃ treatment if occlusion did not occur (>10x the time for WT carotid artery thrombosis). The α-β-methylene-ADP compound (Cat# M3763 Sigma-Aldrich) for in vivo thrombosis was used at the concentrations indicated in figure legend.

Isolation of aortic endothelial cells
Mice were sacrificed under sedation (Ketamine: 80mg/kg body weight, Xylazine: 15 mg/kg body weight). Manual collection of the aorta has been described elsewhere⁶, however instead of isolating mononuclear cells, a protocol for endothelial cell isolation was used to obtain aortic endothelial cells for flow cytometric analysis.⁷

Stable hCD39-expressing HEK293 cells
HEK293 cells were grown in DMEM supplemented with 10% fetal calf serum. Cells were transfected with the pUNO1-hCD39a expression plasmid that contains the human CD39 cDNA under the control of the hEF1-HTLV promoter that is selectable with Blasticidin (Invivogen, San Diego, CA) using Polyfect Transfection Reagent (Qiagen, Valencia, CA). At 24 hours following
transfection, cells were selected in growth media containing Blasticidin (10 ug/ml). Isolated clones were obtained and cells maintained in DMEM supplemented with Blasticidin (20 ug/ml).

**CD39 activity assay**
To measure CD39 activity, hCD39 transfected HEK293 cells were seeded at 40,000 cells per well in a 96 well plate. Forty-eight hours after seeding, the cells were assayed for CD39 activity in the absence or presence of increasing concentrations of APCP with a constant final percent DMSO of x% using a modified malachite green assay (SensoLyte MG Phosphate Assay Kit, AnaSpec, Fremont, CA).

**Statistical Analysis**
The results of experiments were analyzed using standard software GraphPad Prism, version 6.0. Results were expressed as the mean ± standard error of the mean. For comparison between 2 groups, significance was determined by an unpaired t-test with Welch’s correction. For comparison of multiple groups, multifactorial ANOVA with post-hoc comparison of the means with Tukey correction was used to determine statistical significance. The specific statistical analysis applied to each experiment is noted in the legend for each figure.

**Materials and Methods References**


Supplemental Figure I. PAR-4 mediated activation of platelets is decreased in hCD39-Tg mice. 

A, Representative FACS histograms showing the binding of P-selectin to diluted platelets from WT and hCD39-Tg mice in response to PAR-4 stimulation. B, Bar graph shows quantification of P-selectin MFI in platelets isolated from WT (n=4) and hCD39-Tg (n=4). C, Representative FACS histograms showing the binding of activated GPαIIbβ3 to diluted platelets from WT and hCD39-Tg mice in response to TRAP-4 stimulation. D, Bar graph shows quantification of activated GPαIIbβ3 MFI in platelets isolated from WT (n=4) and hCD39-Tg (n=4). Bars in scatter plots represent mean ± standard error of the mean. In A and B, P values were determined with an unpaired t-test with Welch’s correction. In D, P values were determined using one-way ANOVA with Tukey correction for multiple comparisons using the WT treatment group at each time point as baseline comparison.
Supplemental Figure II. Effect of APCP on CD39 activity in transfected HEK293 cells. HEK293 cells were transfected with hCD39-cDNA expression plasmid and selected in Blasticidin supplemented media. Expression was confirmed by FACS analysis. A, Representative histograms showing hCD39 expression in transfected HEK293 cells B, Nucleotidase activity in blank plasmid transfected HEK293 cells or hCD39-transfected HEK293 cells treated with increasing APCP concentrations (n=5/dose) in response to 100 µM ATP. Baseline represents HEK cells that received 0 mM APCP and 0 µM ATP. 100% activity was set at maximal in hCD39-transfected HEK293 cells. Bars in graph represent mean ± standard error of the mean. In B, P values were determined by one-way ANOVA with Tukey correction using the 0 mM APCP treatment group as the maximum nucleotidase activity for each cell line.
Supplemental Figure III. Bone marrow transplant does not affect endogenous levels of mouse CD39. A-B, peripheral blood mononuclear cells from WT and lethally irradiated male WT mice reconstituted with WT marrow were analyzed for mCD39 expression in CD45+ leukocytes by FACS. A, Representative histograms showing mCD39 expression and B, graph showing quantification of mean fluorescent intensity in WT (n=5) and WT recipient mice that received WT bone marrow BMT (WT→WT) (n=5), Bars in graph represent mean ± standard error of the mean. In B, P value was determined with an unpaired t-test with Welch’s correction.
Supplemental Figure IV. Expression of hCD39 on endothelium and circulating immune cells in hCD38-Tg mice. A, Representative histograms from FACS measuring hCD39 (red line) versus isotype control (grey line) staining in aortic endothelium (CD31+,CD45−) and T cells (TCRβ⁺), B cells (CD19⁺) and Monocytes (CD11b⁺, Ly6C⁺) all isolated from peripheral blood mononuclear cells. B Graph shows quantification of mean fluorescent intensity (ΔMFI = hCD39 Antibody- isotype antibody) of hCD39 in hCD39-Tg mice (n=3). Bars represent mean ± standard error of the mean. P values were determined using one-way ANOVA with Tukey correction for multiple comparisons.
Supplemental Figure V. Mouse CD39 (mCD39) levels in hCD39-Tg and WT mice. A, Representative histograms from fluorescently activated cell sorting measuring mCD39 (red line) versus isotype control (grey line) staining in aortic endothelium (CD31+,CD45-) and Monocytes (CD11b+, Ly6C+) from peripheral blood mononuclear cells. B Graph shows quantification of mean fluorescent intensity ($\Delta$MFI = mCD39 Antibody- isotype antibody) in hCD39-Tg (n=3) and WT (n=3) mice. Bars represent mean ± standard error of the mean. $P$ values were determined using one-way ANOVA with Tukey correction for multiple comparisons.
Supplemental Figure VI. ENPP1 expression in hCD39-Tg and WT mice. A, Representative histograms from fluorescently activated cell sorting measuring ENPP1 (red line) versus isotype control (grey line) staining in aortic endothelium (CD31⁺,CD45⁻) and Monocytes (CD11b⁺, Ly6C⁺) from peripheral blood mononuclear cells. B Graph shows quantification of mean fluorescent intensity (ΔMFI = ENPP1 Antibody-isotype antibody) in hCD39-Tg mice (n=3) and WT (n=3). Bars represent mean ± standard error of the mean. P values were determined using one-way ANOVA with Tukey correction for multiple comparisons.
Supplemental Figure VII. CD73 expression in hCD39-Tg and WT mice. A, Representative histograms from fluorescently activated cell sorting measuring CD73 (red line) versus isotype control (grey line) staining in aortic endothelium (CD31+,CD45-) and Monocytes (CD11b+, Ly6C+) from peripheral blood mononuclear cells. B Graph shows quantification of mean fluorescent intensity ($\Delta$MFI = CD73 Antibody-isotype antibody) in hCD39-Tg (n=3) and WT (n=3) mice. Bars represent mean ± standard error of the mean. $P$ values were determined using one-way ANOVA with Tukey correction for multiple comparisons.
Arterial Injury

- Normal levels of CD39 → Vessel occlusion
- Increased levels of CD39 in endothelium → Vessel occlusion
- Increased levels of CD39 in the immune system → Vessel patency
  - Depletion of monocytes with increased levels of CD39 → Vessel occlusion