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The Endothelial Cell Ecto-ADPase Responsible for Inhibition of Platelet Function is CD39


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Abstract

We previously demonstrated that when platelets are in motion and in proximity to endothelial cells, they become unresponsive to agonists (Marcus, A.J., L.B. Safier, K.A. Hajjar, H.L. Ullman, N. Islam, M.J. Broekman, and A.M. Eiroa. 1991. J. Clin. Invest. 88:1690–1696). This inhibition is due to an ecto-ADPase on the surface of endothelial cells which metabolizes ADP released from activated platelets, resulting in blockade of the aggregation response. Human umbilical vein endothelial cells (HUVEC) ADPase was biochemically classified as an E-type ATP-diphosphohydrolase. The endothelial ecto-ADPase is herein identified as CD39, a molecule originally characterized as a lymphoid surface antigen. All HUVEC ecto-ADPase activity was immunoprecipitated by monoclonal antibodies to CD39. Surface localization of HUVEC CD39 was established by confocal microscopy and flow cytometric analyses. Transfection of COS cells with human CD39 resulted in both ecto-ADPase activity as well as surface expression of CD39. PCR analyses of cDNA obtained from HUVEC mRNA and recombinant human CD39 revealed products of the same size, and of identical sequence. Northern blot analyses demonstrated that HUVEC express the same sized transcripts for CD39 as MP-1 cells (from which CD39 was originally cloned). We established the role of CD39 as a prime endothelial thromboregulator by demonstrating that CD39-transfected COS cells acquired the ability to inhibit ADP-induced aggregation in platelet-rich plasma. The identification of HUVEC ADPase/CD39 as a constitutively expressed potent inhibitor of platelet reactivity offers new prospects for antithrombotic therapeutics. (J. Clin. Invest. 99:1351–1360.) Key words: thrombosis • platelet aggregation • ATPase • apyrase • ATP-diphosphohydrolase • ecto-nucleotidase

Introduction

Platelet activation, a consequence of vascular injury, is counteracted by an antithrombotic endothelial cell response which results in limitation or reversal of the potentially occlusive effects of platelet accumulation (1, 2). Data from our laboratory have demonstrated that platelets become unresponsive to agonists when in proximity to endothelial cells (3–5). This inhibition of platelet responsiveness is due to at least three separate thromboregulatory systems: eicosanoids, endothelium-derived relaxing factor (nitric oxide, NO), and an ecto-nucleotidase on endothelial cells which metabolizes the released platelet agonist, ADP (2). Removal of ADP eliminates platelet recruitment and results in return of platelets to the resting state. ADP-induced platelet reactivity and recruitment represents the final common pathway leading to formation of an occlusive thrombus (1). Thus, the endothelial ecto-ADPase is a critical component of thromboregulation.

Our preliminary studies indicated that the human umbilical vein endothelial cell (HUVEC) ADPase was a membrane-associated ecto-nucleotidase of the E-type (6). This was verified by its Ca/Mg dependence, the ineffectiveness of specific inhibitors of P-, F-, and V-type ATPases, and the capacity to metabolize both ATP and ADP, but not AMP. These characteristics identify the HUVEC enzyme as an apyrase (ATP diphosphohydrolase, ATPDase, EC 3.6.1.5 (6, 7). Research on ecto-nucleotidases had been encumbered by difficulties in isolation, which may have been due to their low abundance and sensitivity to denaturing agents (6, 8–10).

Recently a soluble apyrase was purified from potato tubers, and its cDNA cloned (11). Sequence analysis revealed 25% amino acid identity and 48% amino acid homology with human CD39 (11), a lymphoid cell activation antigen (12). CD39, a cell-surface glycoprotein, is expressed on activated NK cells, B cells, and subsets of T cells, as well as some HUVEC cell lines (13). Based on these reports we hypothesized that HUVEC ADPase shares homology with CD39. Experimental data described herein demonstrate identity between CD39 and HUVEC ecto-ADPase.

Methods

Isolation and culture of HUVEC. Endothelial cells from human umbilical veins were grown to confluence in medium M199 (Mediatech, Herndon, VA) with added glucose, penicillin, streptomycin, and 20% FBS (Sigma Chemical Co., St. Louis, MO) and used at passage 2–4 (3, 4). Cells were studied in monolayer, or detached with collagenase-EDTA, washed and resuspended in Hepes-buffered saline (HBS; 0.14 M NaCl, 5 mM KCl, 15 mM Hepes, pH 7.4) (3, 4).

1. Abbreviations used in this paper: ASA, acetylsalicylic acid, aspirin; ATPDase, ATP diphosphohydrolase; HBS, Hepes-buffered saline; HUVEC, human umbilical vein endothelial cells; Triton, Triton X-100; TSG, Tris-buffered saline.

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**Subcellular fractionation procedure for HUVEC ADPase.** Washed HUVEC monolayers were harvested with collagenase-EDTA, washed twice in HBS and treated with 2 mM diisopropylfluorophosphate in HBS (5 min, 4°C), followed by two more washes (14, 15). Cells were sonicated (10 s, 3–5 times), centrifuged (150 g, 10 min), and supernatants differentially centrifuged (10,000 g, 15 min, followed by 100,000 g, 1 h) (14, 15). The 10,000 g and 100,000 g pellets were resuspended in Tris-buffered saline (TSG) buffer (15 mM Tris, 134 mM NaCl, 5 mM glucose, pH 7.4, containing 10% ethylene glycol, 0.5% Triton X-100, followed by ultracentrifugation (100,000 g, 90 min). Pellets and final supernatants were examined for protein content, and assayed for ADPase activity by a sensitive and specific procedure which measures metabolism of \([^{3}P]ADP\) (3). This subcellular fractionation and solubilization procedure was also used with transfected COS cells.

**Assay for ADPase activity.** Cell suspensions or monolayers were incubated with 50 μM \([^{3}P]ADP\) in 150 μL assay buffer (15 mM Tris, 134 mM NaCl, 5 mM glucose, pH 7.4, containing 10 μM AP5a (P2, P3-diadenosine-5’-pentaphosphate), 1 mM ouabain, 10 μM dipryridamole, and 3 mM CaCl2) for 5 min (37°C). Suspended cells were removed by centrifugation (10 s, 15,600 g). 100 μL supernatant was added to 10 μL “stop solution” (160 mM disodium EDTA, pH 7.0, 17 mM ADP, 0.15 M NaCl) at 4°C to block further metabolism of ADP (3). Nucleotides, nucleosides and bases were separated by TLC using specific procedure which measures metabolism of \([^{3}P]ADP\) (3). This subcellular fractionation and solubilization procedure was also used with transfected COS cells.

**RT-PCR.** Total RNA was prepared by the guanidinium thiocyanate/CS2 gradient method (16). HUVEC mRNA was prepared using the mRNA Direct kit (Dynal Inc., Lake Success, NY). First strand cDNA was synthesized from total RNA or mRNA by oligo(dT)-priming, pHuCD39 (12) or first-strand cDNA (0.1 ng) was PCR amplified using AmpliTaq polymerase (Perkin-Elmer Corp., Branchburg, NJ) and HuCD39 cDNA-specific primer pairs. The following primer pairs were used: 5’ GC TTC AAC AAAT TAC TCT AG3’ (sense primer No. 9032, corresponding to nucleotides 165–122 (12), GenBank accession No. S78513) and 5’ CTT TCC CAT CTC GAG A A C3’ (antisense primer No. 8938, nucleotides 489–472). For this primer pair, annealing temperature was 58°C and size of the PCR product 385 bp. Sense primer No. 9032 (above) and 5’ CCG GAG AT T T C A T A C3’ (antisense No. 8868, nucleotides 904–888). For this primer pair, annealing temperature was 53°C and PCR product size 800 bp. 5’ GTG TGC CAG GGT GAA G3’ (sense No. 8939, nucleotides 472–489) and 5’ TAG GTT TCA A TAC C C C C3’ (antisense No. 9035, nucleotides 1032–1016). For this primer pair, annealing temperature was 57°C, and PCR product size 561 bp. 5’ TAG CTT CCT GT G C T A G3’ (sense, No. 9034, nucleotides 820–836) and 5’ AAG GCT GAC C A G A A C3’ (antisense, No. 8817, nucleotides 1247–1231). For this primer pair, annealing temperature was 57°C, and PCR product size 428 bp. PCR amplification was performed using 30 cycles of denaturation at 94°C for 45 s, specified annealing temperature for 45 s, and extension at 72°C for 1 min, followed by a finishing cycle at 72°C for 6 min. PCR reaction mixtures consisted of 1 μM of each primer oligo, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.001% (wt/vol) gelatin, 200 μM of each deoxynucleotide triphosphate (dNTP), and 0.05 U/μl AmpliTaq polymerase (50 μl final volume). PCR products were separated by electrophoresis in 2% MetaPhor agarose (FMC Bioproducts, Rockland, ME) in Tris-borate EDTA buffer (16). DNA was stained with ethidium bromide and visualized by UV transillumination. PCR fragments were sequenced by the primer walking technique (17) (Taq-FS DyeDeoxy™ terminator cycle sequencing kit and ABI Prism 377 DNA Sequencer; Perkin-Elmer Corp.).

**Northern blot analyses.** Total (5 μg) and poly(A) (1 μg) RNA were separated by formaldehyde-agarose gel electrophoresis, transferred to Hybond-N (Amersham, Arlington Heights, IL) by capillary flow (16), and hybridized with single-stranded HuCD39°°°ºº PCR probe. The probe was generated from pHuCD39 by PCR using a Bgl II/AscI cloning site, and conjugated with biotin (Roche Diagnostics, Mannheim, Germany) before autoradiography.
Confocal microscopy. HUVEC or COS-1 cells transfected with pHuCD39 or control vector pDC303 as described (12) were grown on coverslips, washed with PBS and fixed with 3% paraformaldehyde (30 min, room temperature). Autofluorescence was quenched with 50 mM NH₄Cl (10 min). Normal goat serum (5% in PBS) was used to block non-specific binding, and where indicated Triton (0.1%, 1 h) was used to permeabilize cells. Cells were then incubated with mAb73 antibody (5 μg/ml in PBS, 5% normal goat serum, 0.1% Triton, 1 h, room temperature), washed 3 times (PBS, 5% normal goat serum, 0.1% Triton), and incubated (1 h, room temperature) with 5 μg/ml goat anti-mouse antibody, labeled with Texas Red, as well as with 10 mM YOYO (nuclear stain). After washing three times as above, cells were mounted in 100 μg/ml DABCO in 50% glycerol. Cells were examined by laser scanning confocal microscopy (Multiprobe 2001; Molecular Dynamics, Sunnyvale, CA). One image of the CD39 staining (Texas Red) was collected, followed by a second image of the cell nuclei (YOYO).

Indirect immunofluorescence analyses. Adherent cells were harvested with trypsin/EDTA and washed in Hanks buffered saline with 1% fetal calf serum/0.01% sodium azide (wash buffer). Cells were then incubated with primary antibody (2A, HC1, 10H83, A1, mAb73, anti-HLA-DR or anti-HLA-ABC) at saturating concentrations (60 min, 4°C), washed three times, and incubated with secondary antibody (goat anti-mouse IgG F(ab)²), labeled with FITC (60 min at 4°C). They were washed twice, resuspended and analyzed on a EPICS cytofluorograph (Coulter Corp., Miami, FL). Where specified, HUVEC were treated with 300 U/ml IFN (R&D Systems, Minneapolis, MN), which was added to the culture medium 18 h before harvesting.

Preparation of platelet-rich plasma. After obtaining informed consent, blood was collected via plastic tubing from volunteers using acid citrate-dextrose (citric acid, 38 mM; sodium citrate, 75 mM; glucose, 135 mM) as anticoagulant (3, 18). Volunteers had ingested 650 mg acetylsalicylic acid, aspirin (ASA) 18 h before blood donation. Platelet-rich plasma (PRP) was prepared with an initial whole blood centrifugation (200 g, 15 min, 25°C), and a second centrifugation of the PRP (90 g, 10 min) to eliminate any residual erythrocytes and leukocytes. The stock suspension of PRP was maintained at room temperature under 5% CO₂-air (3).

Aggregation experiments with combined suspensions of ASA-treated platelets and HUVEC or CD39-transfected COS cells. ASA-PRP containing 1.22 × 10⁶ platelets was preincubated (3 min, 37°C) in an aggregometer cuvette (Lumiaggregometer; Chrono-Log, Havertown, PA) alone or in combination with other cells: HUVEC (1.7 × 10⁶), or 3.68 × 10⁵ MP-1 cells, pHuCD39- or pMuCD39-transfected COS cells or COS cells transfected with pDC303 (vector alone) (12). Platelet-poor plasma, with the same number of HUVEC or other cells added, was used as a blank in order to correct for light absorption by these cells. Total volumes were adjusted to 300 μl with TSG buffer. After the 3-min preincubation, ADP was added at the concentration indicated, and the aggregation response recorded for 4–5 min.

Materials. EBV-transformed B lymphoblastoid cell lines (B-LCL) 9053 and 9055 ( homozygous HLA tissue typing lines) were grown in RPMI/10% fetal calf serum. MRC5 fibroblasts (ATCC, Rockville, MD) were used at passage 10–20. MP-1 cells were cultured as described (12). Jurkat, Daudi, and COS cells were obtained from ATCC.

Antibodies used were 2A, specific for a mycoplasma protein (negative control); HC1, specific for endothelial cells (19); 10H83, specific for ICAM-CD54; A1 (Zymed Laboratories Inc.), AC1 (Immunotech, Westbrook, ME), and mAb73 (12) specific for CD39 (generously provided by Dr. Guy Delespesse, University of Montreal); purified mouse myeloma IgG, (Zymed Laboratories Inc.); anti-HLA-DR (VG2.1, courtesy of Dr. S.M. Fu, University of Virginia); anti-HLA-ABC (Olympus, Melville, NY).

Reagents. Texas Red, YOYO (Molecular Probes Inc., Eugene, OR); PI-specific phospholipase C (Boehringer Mannheim, Indianapolis, IN); concanavalin A, DABCO (1,4 diazabicyclo[2.2.2] octane), phenylmethylsulfonylfluoride, leupeptin, pepstatin A, disopropylfluorophosphate, ouabain, dipyridamole, P₂,₃-di(adenosine-5'-) penta-phosphate (Sigma Chemical Co.); [³²P]ADP (DuPont-NEN, Wilmington, DE). All other chemicals were molecular biology grade or equivalent.

Results

Biochemical properties of HUVEC ADPase. ADPase activity was previously demonstrated on the luminal surface of HUVEC in tissue culture (3). The enzyme was partially purified by solubilization with 0.5% Triton and 10% ethylene glycol (see Methods). Further ADPase purification was achieved by MonoQ Sepharose chromatography (see Methods, Fig. 1). To
establish whether HUVEC ADPase was an E-type ATP diphosphohydrolase, we studied its biochemical properties. Enzyme activity of the MonoQ ADPase preparation was enhanced by Ca\(^{2+}\) and Mg\(^{2+}\), and inhibited by EDTA (Table I).

In additional experiments, treatment of HUVEC monolayers with PI-specific phospholipase C had no effect on HUVEC enzyme activity, indicating that the ADPase was not GPI-anchored (data not shown). Triton solubilization of HUVEC membranes resulted in separation of ADPase from alkaline phosphatase. Moreover, concanavalin A increased enzyme activity twofold, indicating that the enzyme is a glycoprotein.

Table I. Divalent Cation Dependence of HUVEC ADPase Activity

<table>
<thead>
<tr>
<th>Divalent cation added</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Ca(^{2+}) (3 mM)</td>
<td>164</td>
</tr>
<tr>
<td>Mg(^{2+}) (3 mM)</td>
<td>122</td>
</tr>
<tr>
<td>EDTA (1 mM)</td>
<td>8</td>
</tr>
</tbody>
</table>

Activity (averages of duplicate measurements) is expressed as percent of activity of MonoQ-purified HUVEC ADPase without added divalent cations in the standard assay (Methods). 100% was 116 pmol/min/µg protein. The data are representative of four or more similar experiments.

Table II. Enzyme Characteristics of HUVEC ADPase Activity

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ouabain (1 mM)</td>
<td>94</td>
</tr>
<tr>
<td>Ap5A* (10 µM)</td>
<td>108</td>
</tr>
<tr>
<td>NEM† (10 mM)</td>
<td>90</td>
</tr>
<tr>
<td>Tetramisole (5 mM)</td>
<td>106</td>
</tr>
<tr>
<td>Sodium azide (1 mM)</td>
<td>87</td>
</tr>
<tr>
<td>Sodium azide (10 mM)</td>
<td>56</td>
</tr>
<tr>
<td>AMP (150 µM)</td>
<td>64</td>
</tr>
<tr>
<td>ADP-β-S (150 µM)</td>
<td>46</td>
</tr>
</tbody>
</table>

Activity (averages of duplicate measurements) is expressed as percent of activity of MonoQ-purified HUVEC ADPase in the presence of 3 mM Ca\(^{2+}\) in the standard assay (Methods). 100% was 211 pmol/min/µg protein. The data are representative of three or more similar experiments. *Ap5A, P\(^1\),P\(^5\)-di(adenosine-5')-pentaphosphate; †NEM, N-ethylmaleimide; §ADP-β-S, adenosine 5’-O-(2-thiodiphosphate).

Figure 2. Immunoprecipitation of HUVEC ADPase activity by CD39 antibodies. (A) Immunoprecipitates were prepared from a precleared HUVEC lysate, using anti-CD39 antibodies A1 and AC2, as well as control IgG1, and assayed for ADPase activity (Methods). (B) HUVEC ADPase, partially purified by MonoQ chromatography (Methods) was immunoprecipitated with antibodies A1 and mAb73, and ADPase activity in supernates (horizontally striped bars) and pellets (solid bars) determined. For comparison, the protein content of the precleared HUVEC lysate (A) or the precleared partially purified preparation (B), were used for normalization of ADPase activity. Note different scales. These data also demonstrate that the ADPase is more than 250-fold purified by the procedure. (C) Partially purified HUVEC ADPase and solubilized membranes from COS cells transfected with pHuCD39 were subjected to serial immunoprecipitation using anti-CD39 (mAb73) or anti-ovalbumin conjugated to Affigel beads. ADPase activity in the supernate was determined at each step and expressed as a percentage of activity in the starting material.
Inhibition of Platelet Function by Endothelial Cell Ecto-ADPase/CD39

Tact HUVEC were incubated with the anti-CD39 antibodies A1 and AC2. The resulting immunoprecipitates had ADPase activity of 85 and 75 pmol/min/mg lysate protein respectively, whereas no ADP was metabolized by the isotype matched control precipitate (Fig. 2A).

In additional experiments, monoclonal antibodies mAb73 and A1 specifically immunoprecipitated HUVEC ADPase activity from a partially purified preparation of the enzyme (Fig. 2B). The ADPase activities of the immunoprecipitates (Fig. 2, A and B) indicated that the initial purification procedures, including the MonoQ chromatography step, resulted in a 250-fold purification relative to total HUVEC cell lysates. Importantly, HUVEC ADPase was immunoprecipitated by three separate antibodies to human CD39.

Greater than 95% of ADPase activity was removed from the purified HUVEC preparation by serial immunoprecipitation with antibody mAb73 (Fig. 2C). Similar immunoprecipitation data were obtained with solubilized membrane preparations from pHuCD39-transfected COS cells. No significant ADPase activity was immunoprecipitated with an irrelevant antibody, anti-ovalbumin (Fig. 2C). Thus, serial immunoprecipitation of CD39 removes all ADPase activity from HUVEC ADPase preparations. This indicates that CD39 is responsible for more than 95% of HUVEC ecto-ADPase activity.

Transfection of COS cells with CD39 confers ecto-ADPase activity. To further develop our hypothesis that HUVEC ADPase is CD39, COS cells were transiently transfected with either pHuCD39, pMuCD39, or control vector pDC303 alone (12). Transfected cells were assayed for ecto-ADPase activity as intact monolayers 48 h later. pHuCD39- and pMuCD39-transfected COS cell monolayers metabolized 50 μM ADP to AMP within 3 min of incubation. The acquired ADPase activity was comparable to or greater than that of HUVEC monolayers (Fig. 3). In contrast, COS cells transfected with vector alone were devoid of ADPase activity (Fig. 3). We concluded that the ecto-ADPase activity of CD39-transfected COS cells is similar to that of HUVEC.

Microsomal membranes from transfected COS cells display ADPase activity. To establish additional similarities between ADPase activity of COS cell transfectants and that of HUVEC, transfectants and HUVEC were processed in parallel, using our partial purification procedure. After homogenization and differential centrifugation, microsomal membranes were solubilized (see Methods). ADPase activity in particulate and solubilized membranes were compared. Comparable percentages of activity were solubilized by Triton X-100 from HUVEC, COS cell transfectants, and HUVEC (Fig. 4). These results support the hypothesis that the ADPase activity of CD39-transfected COS cells is similar to that of HUVEC ADPase.

HUVEC mRNA and HuCD39 cDNA yield RT-PCR products of equal size. Four different primer pairs (9032/8938, 9032/8868, 8939/9035, and 9034/8817), spanning the NH₂-terminal 75% of the CD39 coding sequence (12), showed identical PCR product sizes between human CD39 and HUVEC mRNA-derived cDNA. Sequence analyses demonstrated 100% identity of the PCR fragments derived from HUVEC mRNA with the published CD39 sequence. PCR template key: H, first-strand cDNA synthesized by RT from HUVEC mRNA; +, HuCD39 cDNA.
and pHuCD39- or pMuCD39-transfected COS cells (Fig. 4). These data confirm the membrane localization of HUVEC ADPase and support the concept that it is identical to CD39.

Comparison of HUVEC mRNA with recombinant human CD39 cDNA. HUVEC mRNA was analyzed by RT-PCR using four separate primer pairs derived from the original sequence of human CD39 (12) with emphasis on its NH₂-terminal portion (postulated to be the enzymatic domain (11, 12)). pHuCD39 cDNA was used for direct comparison of PCR product sizes. The data (Fig. 5) demonstrated PCR products of similar size between HUVEC and pHuCD39 for each of the four fragments spanning 1144 of the 1529 bp of the coding sequence of human CD39. Sequence analyses of gel-purified PCR products confirmed 100% identity between endothelial cell CD39 and the published CD39 sequence (data not shown).

Northern blot analyses using a probe derived from HuCD39 cDNA (Fig. 6) revealed that mRNA for CD39 in HUVEC was expressed in the same band pattern as in MP-1 cells, an EBV-transformed B cell line from which CD39 was originally cloned (12). No message was detected in Jurkat, Daudi, or COS cells transfected with pDC303 (vector alone). COS cells transfected with pHuCD39 showed a single positive band, conforming to the 1.9 kb cloned species (12). The positions of 28 S and 18 S rRNA are indicated by the dashed lines for both human and simian rRNAs.

Figure 6. Expression of CD39 RNA in HUVEC. Poly(A) and total RNA were separated by formaldehyde gel electrophoresis, transferred to Hybond N membrane and hybridized with a 3²P-labeled probe derived from the 5' region of HuCD39 cDNA (Methods). The size of the messages for CD39 in HUVEC (EC) was identical to that in MP-1 cells (from which CD39 was originally cloned) (12). No message was detected in Jurkat, Daudi, or COS cells transfected with pDC303 (vector alone). COS cells transfected with pHuCD39 showed a single positive band, conforming to the 1.9 kb cloned species (12). The positions of 28 S and 18 S rRNA are indicated by the dashed lines for both human and simian rRNAs.

Figure 7. Confocal microscopy demonstrates CD39 binding to HUVEC at the cell surface. HUVEC were stained with mAb73 anti-CD39 (A) or IgG1 isotype control (C), then with goat anti-mouse IgG labeled with Texas Red. The same cells were counterstained with YOYO to highlight nuclei (B and D). Bar, 10 μm.
Plasma membrane localization of HUVEC CD39. Confocal microscopy demonstrated the presence of CD39 on the surface of HUVEC and pHuCD39-transfected COS cells. Fig. 7 depicts staining of HUVEC with anti-CD39 antibody in a pattern indicative of membrane localization of the antigen. Anti-CD39 bound specifically to HUVEC (Fig. 7 A), with isotype control showing only background staining (Fig. 7 C). CD39-specific staining was most intense at the cell surface. Fig. 7, B and D demonstrates counterstaining of the nuclei of the cells shown in panels A and C. Confocal microscopy of COS cells transfected with pHuCD39 or vector alone verified the presence of CD39 both at the cell membrane and intracellularly in pHuCD39 transfectants but not in COS cells transfected with control vector (Fig. 8).

Flow cytometric analyses demonstrated that HUVEC at passage 2 stained positively with both CD39 antibodies (A1, mAb73) with a 20-fold increase in intensity over background (Fig. 9). Consistent with a previous report, B-LCL cells also stained positively, but the MRC5 fibroblast cell line did not (13). Moreover, trypsin/EDTA, used to harvest adherent HUVEC and fibroblasts before staining, had no effect on expression of CD39 epitopes on the B-LCL cells. The endothelial origin of the HUVEC was confirmed by HC1 staining (19). The lack of HLA-DR expression indicated that the HUVEC were in a resting state, since activated HUVEC (e.g., γIFN-treated) are induced to express HLA-DR (20). When HUVEC were treated with γIFN (300 U/ml, 18 h) there was no effect on expression of CD39 (data not shown).

Taken together, the confocal microscopy and indirect immunofluorescence results verified that CD39 was present on the plasma membrane of HUVEC and CD39-transfected COS cells. These observations support the concept of CD39 as the HUVEC ecto-ADPase.

Control of platelet reactivity by CD39. If, as shown above, HUVEC ADPase and CD39 are identical, then CD39-bearing cells should exhibit the biological function of HUVEC ADPase, i.e. blockade or reversal of platelet responsiveness to the agonist ADP. We verified this hypothesis using CD39-expressing cells, both endogenous (HUVEC, MP-1), and COS cells transfected with pHuCD39 or pMuCD39.

COS cells transfected with vector alone (Fig. 10 a), in combined suspension with aspirin-treated platelet-rich plasma, had no effect on the normal platelet response to ADP (ASA-PRP, Fig. 10 b). In contrast, when HUVEC (Fig. 10 d) or COS cells transfected with pHuCD39 or pMuCD39 (Fig. 10, e and f) were combined with PRP, platelet reactivity to 10 μM ADP was reversed within 1 min. Furthermore, MP-1 cells, which express both CD39 antigen (12) and ecto-ADPase activity (data not shown), also inhibited ADP-induced platelet aggregation (Fig. 10 c). The data provide direct evidence that CD39 per se endows cells, which are inherently devoid of platelet inhibitory activity, with the functional properties of HUVEC ecto-ADPase.

Discussion
The endothelial cell ecto-ADPase and CD39 are identical, as indicated by our experimental data. Evidence for this conclusion is summarized as follows: Anti-CD39 antibodies immunoprecipitated more than 95% of ADPase activity from an ADPase preparation purified from endothelial cell mem-

Figure 8. COS cells transfected with pHuCD39 specifically acquire CD39. Confocal microscopy of COS cells transfected with pHuCD39 (A and B) or pDC303 (control vector, C and D) were fixed and examined directly (A and C) and after permeabilization (B and D). Cells were stained with mAb73 anti-CD39 followed by goat anti-mouse IgG labeled with Texas Red. CD39 was detected both at the cell membrane and intracellularly in pHuCD39 transfectants. Anti-CD39 staining was undetectable in control vector transfec-
Figure 9. Indirect immunofluorescence demonstrates the presence of CD39 on the surface of HUVEC. EBV B-LCL, HUVEC and MRC5 fibroblasts were stained with the indicated primary antibodies as described in Methods. The percentage of positive cells and the region counted are indicated in each panel and graphically highlighted by shading. B-LCL results depicted were obtained with cell line 9053; cell line 9055 yielded identical data. The MRC5 fibroblasts served as negative controls.

Figure 10. Blockade and reversal of platelet aggregation to ADP by intact HUVEC, MP-1 cells, and COS cells transfected with full-length CD39. Aspirin-treated platelet-rich plasma (ASA-PRP) was stimulated with 10μM ADP, and the aggregation response measured over a 4-min period. (a) Addition of COS cells, transfected with vector alone, resulted in an aggregation response indistinguishable from (b) ASA-PRP alone. (c) MP-1 cells, the original source for cloning of CD39 (12), reversed the aggregation response. (d) Intact HUVEC reversed the aggregation response to ADP to a slightly greater degree than did MP-1 cells. COS cells transfected with either pHuCD39 (e) or pMuCD39 (f) demonstrated an even greater inhibitory effect on platelet responsiveness than HUVEC, which correlated with their higher ecto-ADPase activity (Fig. 3). Aggregation responses were measured in 250 μl PRP, containing 1.22 × 10^8 platelets, combined with 3.68 × 10^5 COS cells or MP-1 cells in 50 μl TSG; in the case of HUVEC, 1.7 × 10^6 cells in 50 μl TSG were used.
branes (Fig. 2). This indicates that CD39 represents the endo-

thelial ecto-ADPase. Confocal microscopy and indirect immu-
nofluorescence studies demonstrated the presence of CD39 on
the HUVEC cell surface (Figs. 7 and 9). Transfection of COS
cells with pHuCD39 resulted in surface expression of both
CD39 (Fig. 8) and ecto-ADPase activity (Fig. 3). For compara-
tive PCR-based analyses, pHuCD39 or cDNA synthesized
from HUVEC mRNA were used as templates in conjunction
with four different CD39-specific primer pairs. This resulted in
products of identical size (Fig. 5) and sequence in each in-
stance. The PCR-generated products encompassed 75% of the
coding region, including the entire putative apyrase conserved
regions (ACR) (11). Furthermore, Northern analyses demon-
strated that HUVEC and MP-1 cells contain messages for
CD39 of the same size (Figs. 5 and 6). Most importantly, trans-
fection of COS cells with the mammalian expression plasmid
pHuCD39 conferred the ability to block ADP-induced platelet
aggregation (Fig. 10).

Rapid reversal of platelet aggregation was observed with
pHuCD39- or pMuCD39-transfected COS cells (Fig. 10).
Moreover, the transflectants metabolized ADP to AMP within
3 min (Fig. 3). These observations are especially appropriate to
the time frame of events leading to formation of a hemostatic
platelet plug or thrombus. Platelet adhesion to injured suben-
dotheium leads to immediate ADP release and recruitment of
additional platelets to form an occlusive thrombus within
4 min (21). This chronology parallels the time course we ob-
served for platelet inhibition by CD39-expressing cells (Fig.
10) and was commensurate with their respective ADPase ac-
tivities (Fig. 3). These results highlight the importance of
CD39 as a thromboregulator.

Historically, a surface marker on B cells immortalized by
Epstein-Barr virus was identified by Rowe and associates in
1982 (22). Expression of this marker, designated CD39, was
subsequently identified in a variety of cells, including macro-
phages, a group of activated natural killer cells, and several en-
dotheial cell lines (13). In 1994, human and murine CD39
were cloned and characterized as cell surface molecules con-
taining two transmembrane regions, two short cytoplasmic
tails, and a large extracellular loop (12).

Recently, sequence homology was described between puri-
fied potato tuber apyrase and human and murine CD39, as
well as several animal and plant nucleotidases (11). While our
studies were in progress, CD39 was reported to be an ecto-
nucleotidase (23). Postulated physiological function(s) for
CD39 or ecto-nucleotidases have been mainly theoretical (6,
13). Although anti-CD39 monoclonal antibodies could induce
homotypic adhesion in CD39+ B cell lines (13), the results re-
ported herein represent the first direct demonstration of a
physiological function for CD39 as an ADPase, i.e. blockade of
platelet responsiveness to the prothrombotic agonist, ADP.

Ecto-nucleotidases have now been characterized from a di-
versity of tissues and species, ranging from insects to parasites
to plants and to mammals (6, 10, 24, 25). Substrate specificity
of ATPDases varies broadly as individual systems are studied.
For example, in most tissues thus far reported, the ratio of
ADPase to ATPase activity ranges from 0.2–1.4. In the case of
HUVEC, this ratio is 2.0. Thus, the HUVEC enzyme is unique
in its high preference for ADP as substrate. This could repre-
sent evolution of an endothelial mechanism targeted toward
metabolism of prothrombotic platelet-derived ADP in prefer-
tence to ATP, thus controlling excessive platelet accumulation.

Another example of ecto-nucleotidases as a circulatory de-
defense system is the Schistosoma mansoni ATPDase, recently
identified on the surface of this circulatory endoparasite (10).
The enzyme has been partially purified from the tegumental
membranes of the parasite. A polyclonal anti-potato apyrase
antibody cross-reacted with the S. mansoni ATPDase (10).
Schistosomes survive in the mesenteric circulation for long pe-
riods of time, successfully evading host responses including
platelet adhesion and aggregation. In analogy with HUVEC,
the S. mansoni ATPDase may metabolize ADP released from
activated platelets coming into contact with the tegumental
surface of the parasite.

It is interesting to note that ecto-ATPases on the surface of
endothelial and other cells (3, 10, 13, 26) have a counterpart on
the surface of activated B-lymphocytes and other cells of the
immune system (13, 27). The capacity for nucleotide removal
may be an essential function for inflammatory cells. Blockade
of ecto-nucleotidase activity results in loss of antigen recogni-
tion and effector function of T, B, and NK cells (27). Alterna-
tively, the observation that an enzymatic activity is common to
distinct cell types may be a reflection of function(s) which have
not as yet been ascertained.

Our results are particularly pertinent for the concept of
thromboregulation. The three known thromboregulators, eico-
sanoids, EDRF/NO and ecto-ADPase, have important bio-
logical properties which merit consideration for therapeutic
intervention. Whereas aspirin treatment controls the prothrom-
botic action of thromboxane, it also prevents formation of an-
tithrombotic prostacyclin, thus limiting aspirin’s effectiveness.
Although EDRF/NO is an aspirin-insensitive inhibitor of
platelet function, it is inhibited in vitro and in vivo by hemo-
globin after its rapid diffusion into erythrocytes (4, 28, 29).
In contrast, the ecto-ADPase/CD39 is aspirin-insensitive, and
completely inhibits platelet reactivity even when eicosanoid
formation and EDRF/NO production are blocked. The data
presented herein highlight an emerging concept in vascular bi-
ology (3): ADPase/CD39 is an effective physiological and con-
stitutively expressed endothelial cell inhibitor of platelet reac-
tivity. We suggest that ADPase/CD39 offers new opportunities
for antithrombotic therapy.

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