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 therapeusis. (*J. Clin. Invest.* 1997. 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)[1] 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 glutamine, 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).

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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.

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, 0.5 mM DTT, pH 7.4) (3). The microsomal (100,000 *g*) pellet was washed by recentrifugation (100,000 *g*, 60 min), and the washed pellet resuspended in TSG. Microsomal membranes were then solubilized by stirring overnight (4°C) in TSG buffer 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 [^{14}C]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 [^{14}C]ADP in 150 μl assay buffer (15 mM Tris, 134 mM NaCl, 5 mM glucose, pH 7.4, containing 10 μM Ap5A (P^1,P^5-di[adenosine-5′] pentaphosphate), 1 mM ouabain, 10 μM dipyridamole, and 3 mM $CaCl_2$) 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 isobutanol/1-pentanol/ethylene glycol monoethyl ether/NH_4OH/H_2O (90:60:180:90:120) (3). A "buffer blan" was incubated in the absence of cells. Radioactivity was quantitated by radio TLC scanning (RTLC multiscanner; Packard, Meriden, CT). Results were calculated as averages of duplicate to quadruplicate measurements after subtraction of buffer blanks (consistently $< 1\%$ of total radioactivity). Data were expressed as percentage of ADP metabolized or as pmoles ADP metabolized per min.

When subcellular or purified enzyme fractions were studied, the assay was performed using 50 μM [^{14}C]ADP in 50 μl of the above assay buffer, containing 0.05% Triton. Incubation time was 15–30 min (37°C). ATPase activity was determined in a similar manner as ADPase activity, substituting 50 μM [^{14}C]ATP for [^{14}C]ADP. Alkaline phosphatase activity was determined spectrophotometrically (Sigma Chemical Co.).

Purification of HUVEC ADPase. Solubilized EC membrane protein (7–10 mg) was diluted with 20 mM Tris buffer (pH 7.3) containing 10% ethylene glycol (vol/vol) to reduce the Triton concentration to 0.1%, and then applied to a MonoQ HR 10/10 column (Pharmacia Biotech, Piscataway, NJ), which had been equilibrated with 30 mM NaCl in 20 mM Tris/10% ethylene glycol/0.05% Triton/0.5 mM DTT (buffer A). Chromatography was performed at a flow rate of 4 ml/min. The column was then washed with buffer A (10 min) to remove unbound protein. Elution of ADPase activity was accomplished with a gradient of 30 to 500 mM NaCl in buffer A (40 min) and 4 ml fractions collected (see Fig. 1). Residual protein was removed from the column with buffer A containing 1 M NaCl. Fractions were assayed for ADPase activity as described above. Protein content was determined by a micro-Coomassie method (Pierce, Rockford, IL). Fractions were stored at −70°C.

Immunoprecipitation. HUVEC suspensions were washed three times in ice-cold PBS, and lysed with 3 ml single detergent lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton, containing 1 mM phenylmethylsulfonylfluoride [PMSF], 10 μg/ml leupeptin, and 10 μg/ml pepstatin), for 30 min at 4°C with agitation. Subsequent procedures were carried out at 0–4°C. Lysates were cleared by centrifugation (15,600 *g*, 15 min). 750 μl precleared lysate (16), diluted 1:1 with TBS/BSA (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton, 3% BSA), was then incubated with 30 μg anti-CD39 antibody A1, AC2, or control IgG_1 (90 min) with gentle agitation. This was followed by incubation with 100 μl protein G beads (Zymed Laboratories, Inc., South San Francisco, CA) (90 min) and centrifugation (200 *g*, 1 min). Pellets were washed with gentle agitation (5 min) and centrifugation (200 *g*, 1 min): once with TBS/BSA containing 500 mM NaCl, twice with TBS/BSA, and once with Tris buffer (10 mM Tris, 0.1% Triton X-100, pH 7.4). Pellets were finally resuspended in 100 μl Tris buffer, and assayed for ADPase activity.

Precleared MonoQ-purified HUVEC ADPase (200 μl, 16.3 μg protein) was immunoprecipitated with 10 μg antiCD39 antibody A1, mAb73, or control IgG_1, and 30 μl protein G beads as above. Pellets were washed four times with TSG/0.05% Triton without DTT, resuspended in 50 μl of the same buffer, and assayed for ADPase activity.

For serial immunoprecipitation, MonoQ-purified HUVEC ADPase (200 μl, 16.3 μg protein) and solubilized membranes from pHuCD39-transfected COS cells (200 μl, ∼ 5 μg protein) were incubated (2 h, 4°C) with 10 μl Affigel beads conjugated with mAb73 or anti-ovalbumin (control). Immunoprecipitates were obtained by centrifugation (200 *g*, 1 min). Supernatants were incubated with a fresh aliquot of mAb73-conjugated beads, and centrifuged as above. This procedure was repeated once more. The pellets (washed as above), and samples of the supernates from each round of immunoprecipitation were analyzed for ADPase activity.

RT-PCR. Total RNA was prepared by the guanidinium thiocyanate/CsCl 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′GCTCCAAGAATATCCTAG3′ (sense primer No. 9032, corresponding to nucleotides 105–122 (12), GenBank accession No. S73813) and 5′CTTTCCATCCTGAGCAAC3′ (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′CCTGAGAATTTCATTAC3′ (antisense No. 8868, nucleotides 904–888). For this primer pair, annealing temperature was 53°C and PCR product size 800 bp. 5′GTTGCTCAGGATGGAAAG3′ (sense No. 8939, nucleotides 472–489) and 5′TAGTTTCCAATACCCTG3′ (antisense No. 9035, nucleotides 1032–1016). For this primer pair, annealing temperature was 57°C, and PCR product size 561 bp. 5′TAGCTTCTTGTGCTATG3′ (sense, No. 9034, nucleotides 820–836) and 5′AAGGCTGAGCACAGAAC3′ (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 $MgCl_2$, 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 ^{32}P-PCR probe. The probe was generated from pHuCD39 by PCR using a BglII/AccI HuCD39 cDNA fragment with oligo No. 8938 as primer. This yielded an antisense probe of 514 nucleotides. Hybridization was carried out using 2×10^6 cpm probe/ml Starks solution (50% formamide, 5× SSC, 50 mM KH_2PO_4, 2× Denhardt's, 20 mM EDTA, 150 μg/ml denatured salmon testes DNA, 0.1% SDS, and 0.05% n-lauroylsarcosine) for 16 h at 63°C. The hybridized blot was washed in 0.1× SSC/0.1%SDS (1 h, 68°C) 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 NH_4Cl (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 mg/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) for 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 γIFN (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% CO_2-air (3).

Aggregation experiments with combined suspensions of ASA-treated platelets and HUVEC or CD39-transfected COS cells. ASA-PRP containing 1.22×10^8 platelets was preincubated (3 min, 37°C) in an aggregometer cuvette (Lumiaggregometer; Chrono-Log, Havertown, PA) alone or in combination with other cells: HUVEC (1.7×10^6), or 3.68×10^5 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_1 (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, diisopropylfluorophosphate, ouabain, dipyridamole, P^1,P^5-di(adenosine-5′) pentaphosphate (Sigma Chemical Co.); [^{14}C]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

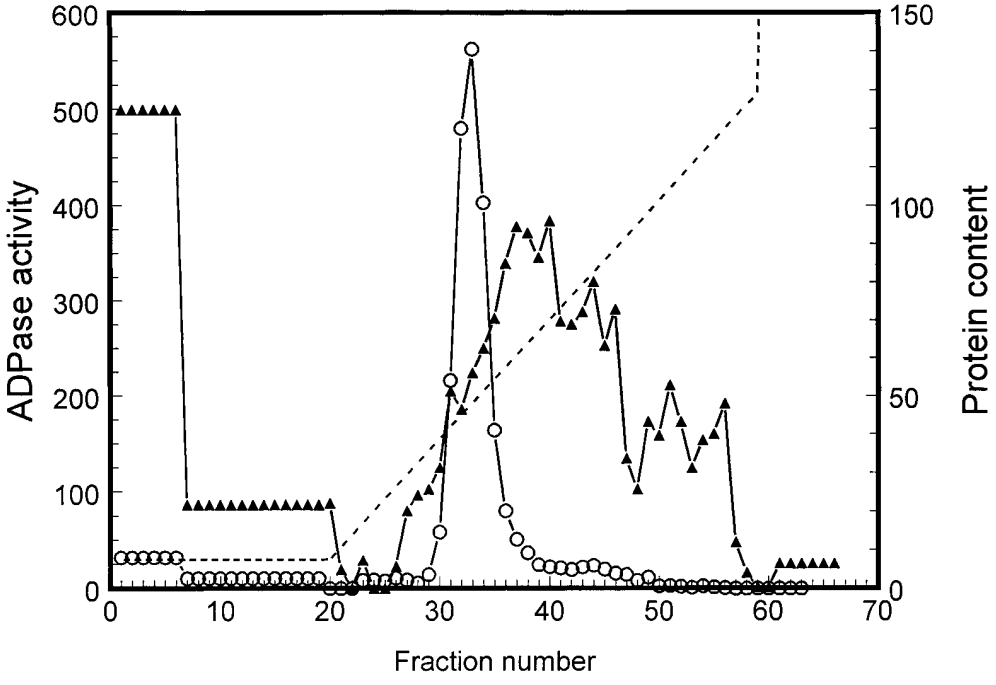

Figure 1. Purification of solubilized HUVEC ADPase by MonoQ Sepharose chromatography. Solubilized HUVEC membranes (7.25 mg protein) were subjected to MonoQ chromatography using a 30 to 500 mM NaCl gradient (*dashed line, left y-axis*). ADPase activity (nmol ADP metabolized per fraction in 30 min, open circles, left *y*-axis) eluted as a single sharp peak at ~ 190 mM NaCl (Methods). Protein (*solid triangles, right y-axis*) is expressed as μg per fraction. Data are representative of more than 10 separate preparations.

Table I. Divalent Cation Dependence of HUVEC ADPase Activity

Divalent cation added	Relative activity (%)
None	100
Ca^{2+} (3 mM)	164
Mg^{2+} (3 mM)	122
EDTA (1 mM)	8

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

Inhibitors	Relative activity (%)
Ouabain (1 mM)	94
Ap5A* (10 μM)	108
NEM‡ (10 mM)	90
Tetramisole (5 mM)	106
Sodium azide (1 mM)	87
Sodium azide (10 mM)	56
AMP (150 μM)	64
ADP-β-S§ (150 μM)	46

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).

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). HUVEC ADPase activity was unaffected by ouabain, Ap5A, *N*-ethylmaleimide, and the alkaline phosphatase inhibitor tetramisole (Table II). In contrast, ADPase activity was inhibited by the substrate analogs ADP-β-S and AMP, and by 10 mM (but not 1 mM) azide (Table II). The ratio of ADPase to ATPase activity in the MonoQ-purified preparation was ~ 2.

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. Based on the above properties, we classified HUVEC ADPase/ATPase as an E-type ATP diphosphohydrolase, EC 3.6.1.5, which is an apyrase, as described in the Introduction.

Monoclonal antibodies to CD39 immunoprecipitate HUVEC ADPase activity. The reported homology between soluble potato tuber apyrase and human CD39 (11), suggested that antibodies against human CD39 would recognize HUVEC ADPase, an apyrase. To test this hypothesis, lysates from in-

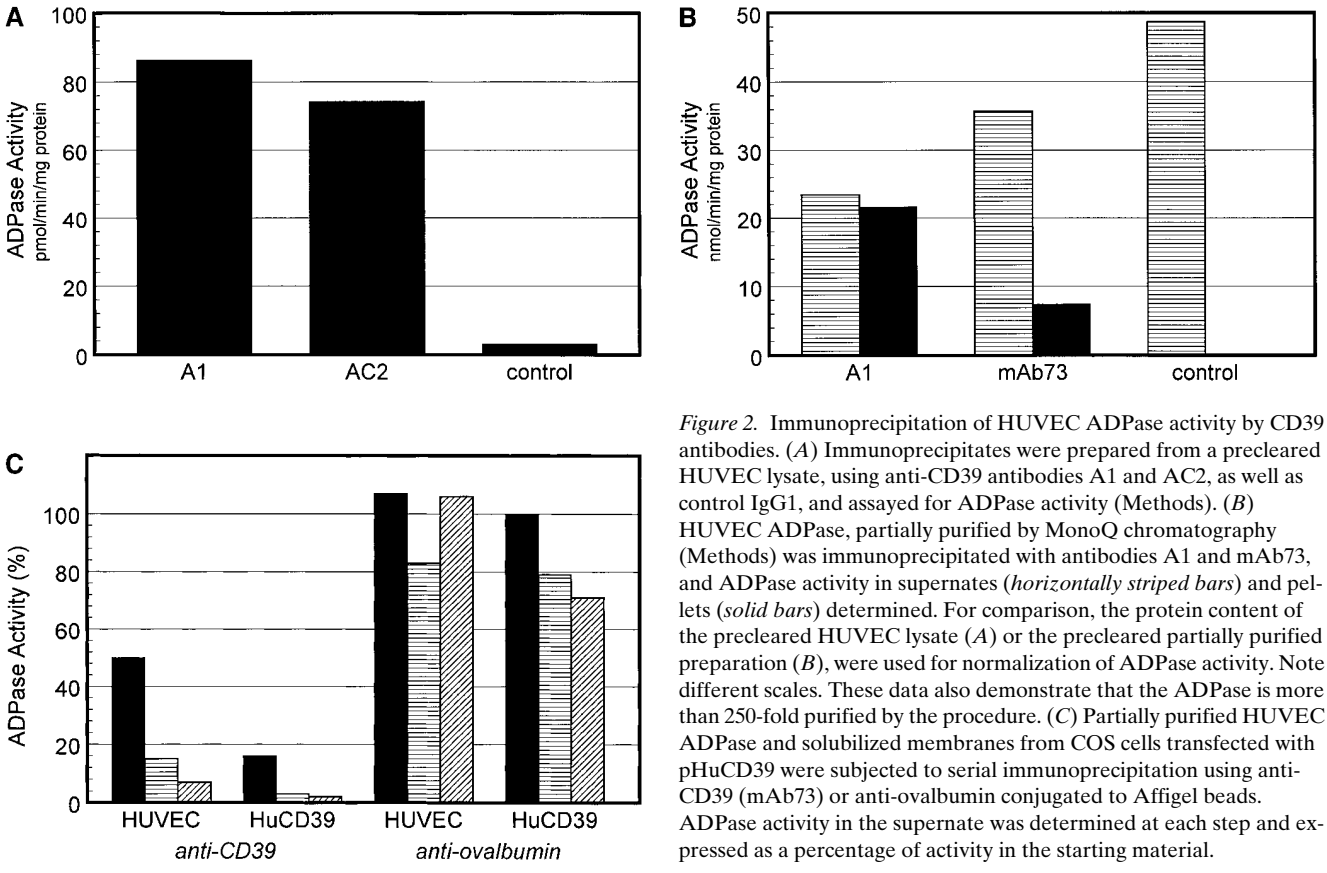

Figure 2. Immunoprecipitation of HUVEC ADPase activity by CD39 antibodies. (*A*) Immunoprecipitates were prepared from a precleared HUVEC lysate, using anti-CD39 antibodies A1 and AC2, as well as control IgG1, and assayed for ADPase activity (Methods). (*B*) HUVEC ADPase, partially purified by MonoQ 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.

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. 2 *A*).

In additional experiments, monoclonal antibodies mAb73 and A1 specifically immunoprecipitated HUVEC ADPase activity from a partially purified preparation of the enzyme (Fig. 2 *B*). 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. 2 *C*). 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. 2 *C*). 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.

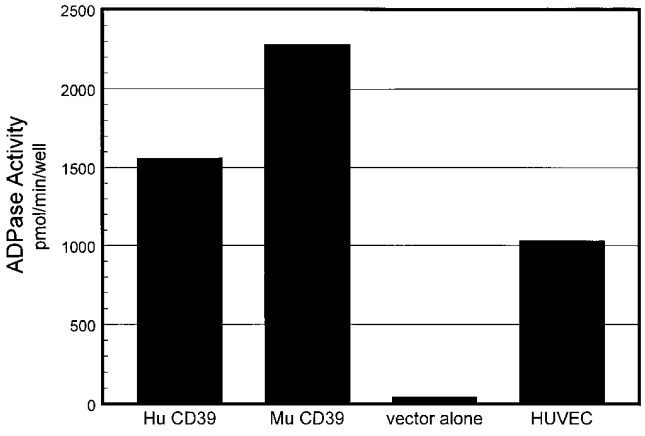

Figure 3. Ecto-ADPase activity of COS cell transfectants in monolayer. Monolayers of COS cell transfectants in 24-well plates were incubated (3 min, 37°C) with ADPase assay medium containing 50 μM ^{14}C-ADP, and ADPase activity determined (Methods). For comparison, HUVEC monolayers were assayed in parallel. COS cells transfected with either pHuCD39 or pMuCD39 acquired ADPase activity comparable to or greater than that of HUVEC monolayers, while the control (vector alone) did not display ADPase activity.

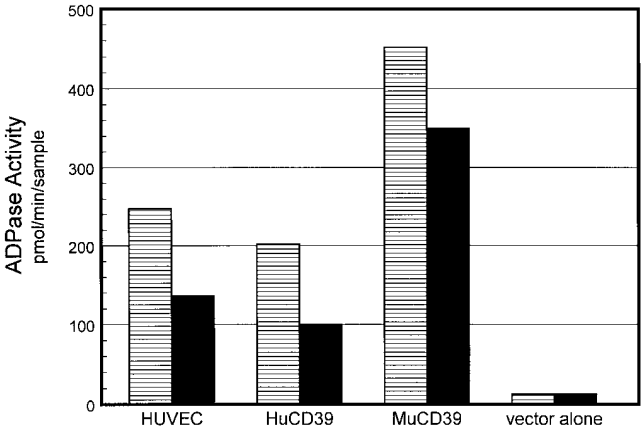

Figure 4. Solubilization of ADPase activity of CD39-transfected COS cell membranes by Triton X-100. Microsomal membranes from HUVEC and COS cells, transfected with pHuCD39, pMuCD39 or vector alone (pDC303), were prepared, solubilized and assayed for ADPase activity (Methods). Hatched bars: ADPase activity of total microsomal membranes; solid bars: activity of solubilized membranes.

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,

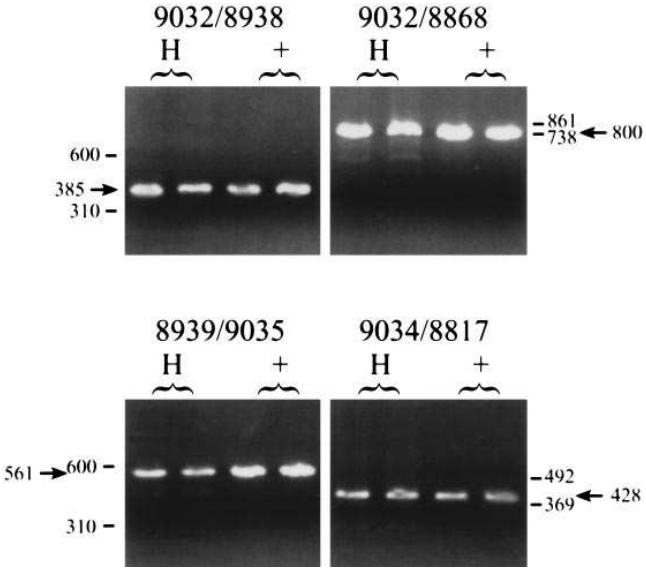

Figure 5. HUVEC mRNA and HuCD39 cDNA yield RT-PCR products of equal size. Four different primer pairs (9032/8938, 9032/8868, 8939/9035, and 9034/8817), spanning the NH_2-terminal 75% of the CD39 coding sequence (12), showed identical PCR product sizes between human CD39 and HUVEC mRNA-derived cDNA. Sequence 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.

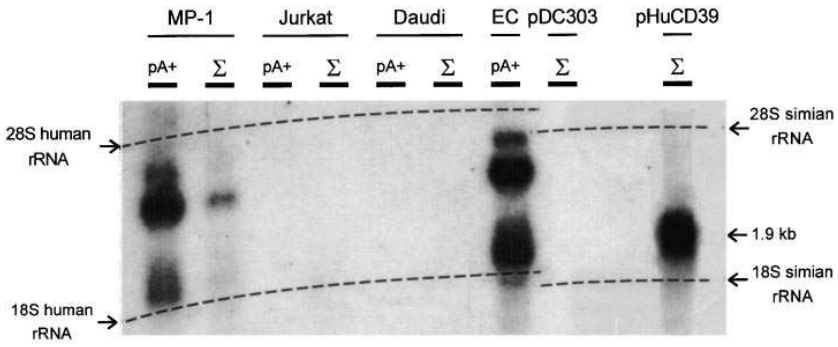

Figure 6. Expression of CD39 RNA in HUVEC. Poly(A) and total RNA were separated by formaldehyde gel electrophoresis, transferred to Hybond N membrane and hybridized with a ^{32}P-labeled probe derived from the 5′ region of HuCD39 cDNA (Methods). The size of the messages for CD39 in HUVEC (EC) was identical 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.

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 NH_2-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 Daudi (non-EBV-transformed B cell line), Jurkat (T cell line), or COS cells transfected with pDC303 (vector alone). As anticipated, the message from pHuCD39-transfected COS cells corresponded to the 1.9-kb cloned species (12).

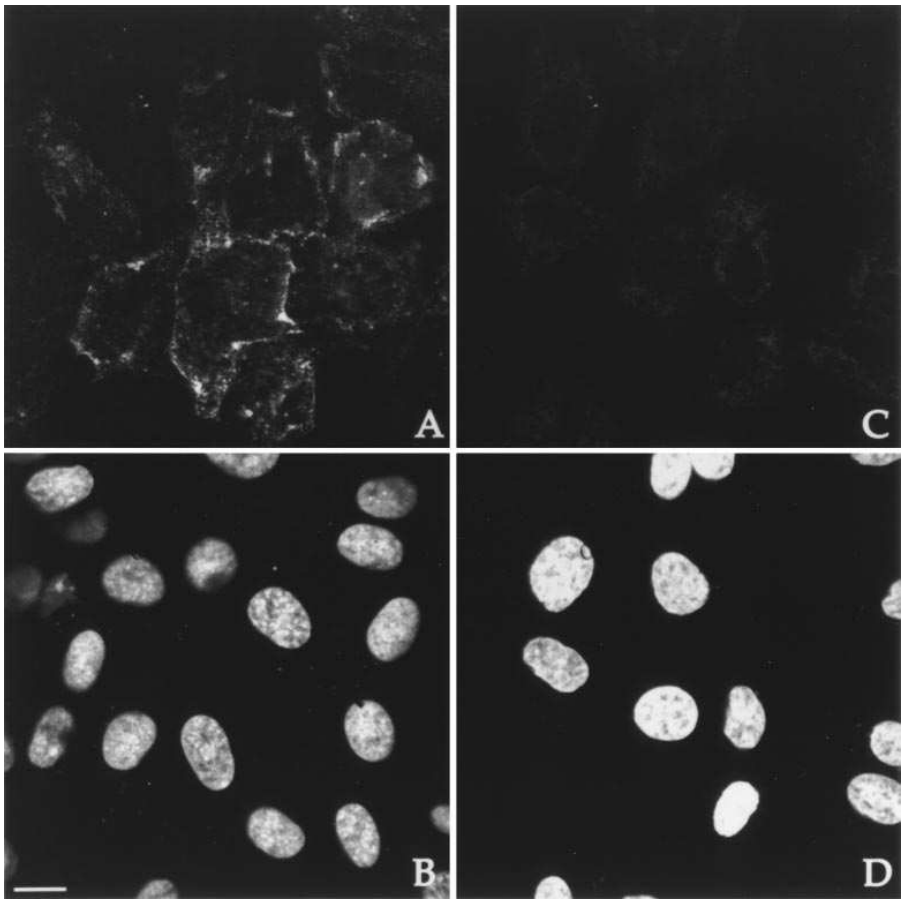

Figure 7. Confocal microscopy demonstrates CD39 binding to HUVEC at the cell surface. HUVEC were stained with mAb73 anti-CD39 (*A*) or IgG1 isotype control (*C*), then with goat anti-mouse IgG labeled with Texas Red. The same cells were counterstained with YOYO to highlight nuclei (*B* and *D*). Bar, 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-

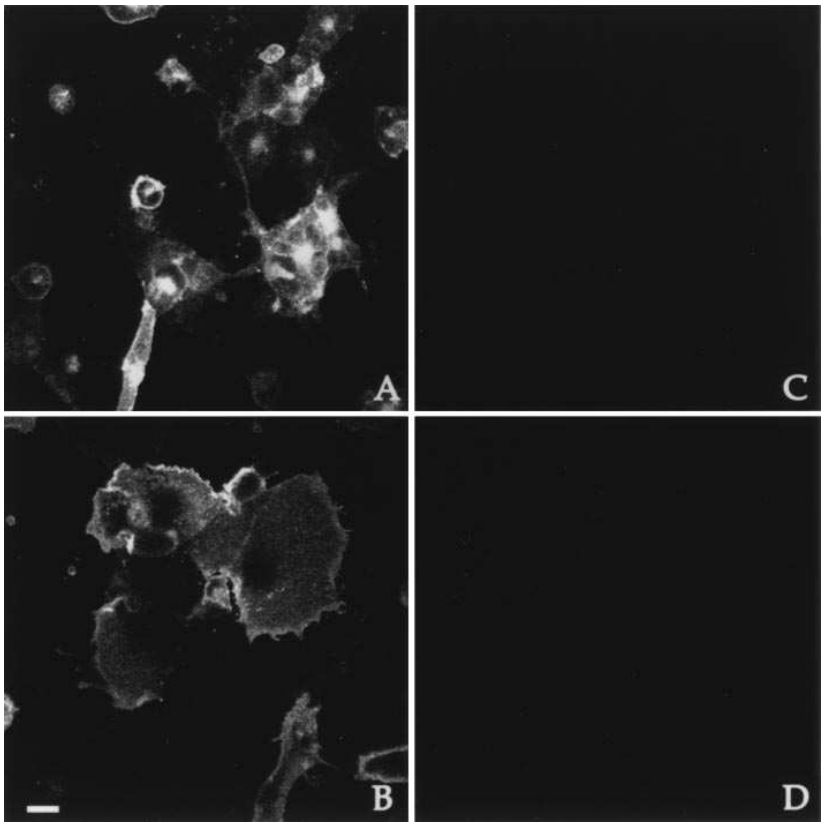

Figure 8. COS cells transfected with pHuCD39 specifically acquire CD39. Confocal microscopy of COS cells transfected with pHuCD39 (*A* and *B*) or pDC303 (control vector, *C* and *D*) were fixed and examined directly (*A* and *C*) and after permeabilization (*B* and *D*). Cells were stained with mAb73 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 transfectants.

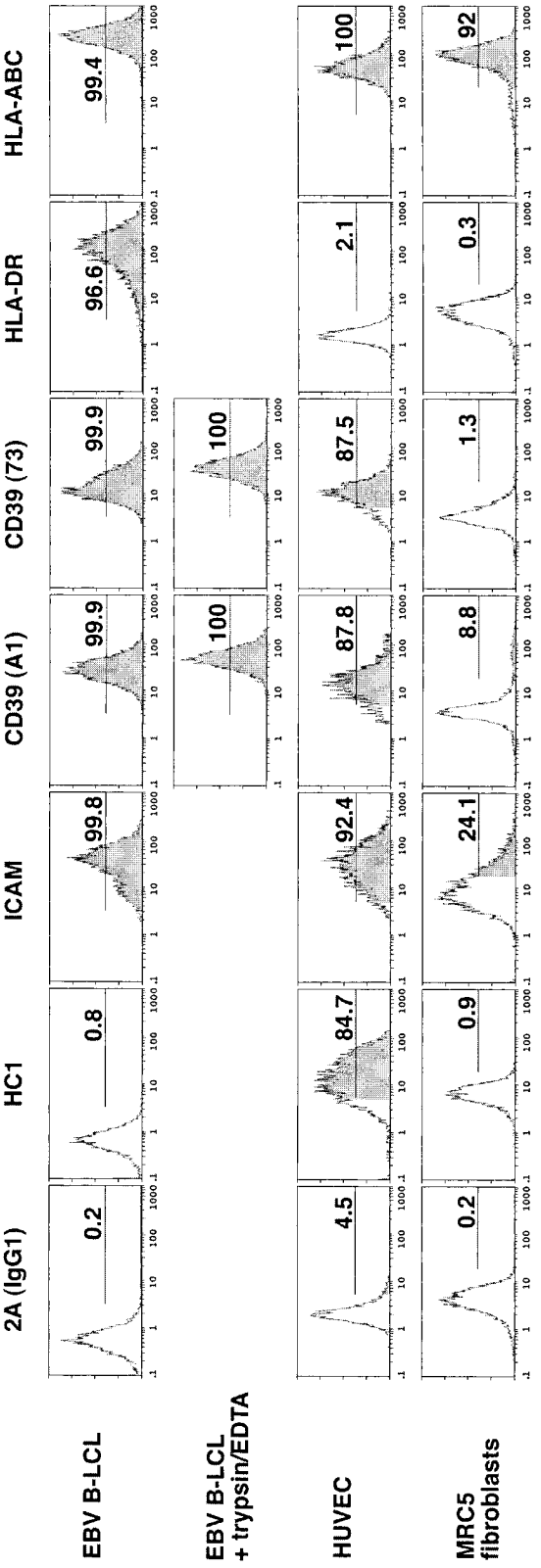

Figure 9. Indirect immunofluorescence demonstrates the presence of CD39 on the surface of HUVEC. EBV B-LCL, HUVEC and MRC5 fibroblasts were stained with the indicated primary antibodies as described in Methods. The percentage of positive cells and the region counted are indicated in each panel and graphically 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.

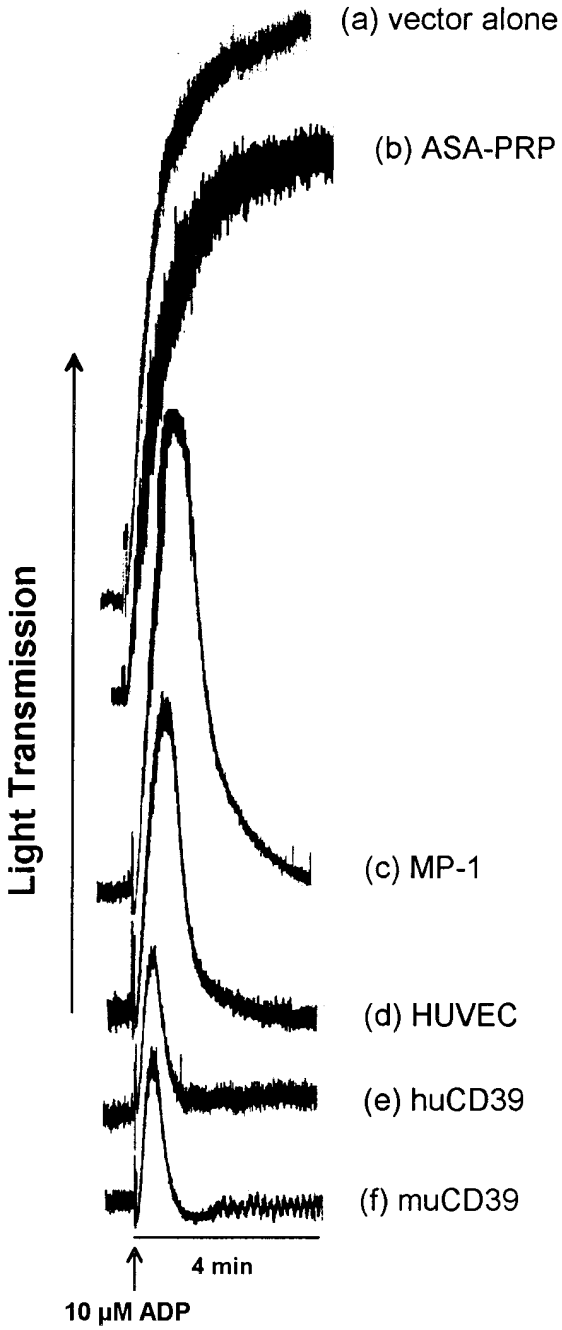

Figure 10. Blockade and reversal of platelet aggregation to ADP by intact HUVEC, MP-1 cells, and COS cells transfected with full-length CD39. Aspirin-treated platelet-rich plasma (ASA-PRP) was stimulated with 10 μM ADP, and the aggregation response measured over a 4-min period. (*a*) Addition of COS 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 endothelial ecto-ADPase. Confocal microscopy and indirect immunofluorescence 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 comparative 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 instance. The PCR-generated products encompassed 75% of the coding region, including the entire putative apyrase conserved regions (ACR) (11). Furthermore, Northern analyses demonstrated that HUVEC and MP-1 cells contain messages for CD39 of the same size (Figs. 5 and 6). Most importantly, transfection 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 transfectants 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 subendothelium 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 observed for platelet inhibition by CD39-expressing cells (Fig. 10) and was commensurate with their respective ADPase activities (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 macrophages, a group of activated natural killer cells, and several endothelial cell lines (13). In 1994, human and murine CD39 were cloned and characterized as cell surface molecules containing two transmembrane regions, two short cytoplasmic tails, and a large extracellular loop (12).

Recently, sequence homology was described between purified 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 reported 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 diversity 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 represent evolution of an endothelial mechanism targeted toward metabolism of prothrombotic platelet-derived ADP in preference to ATP, thus controlling excessive platelet accumulation.

Another example of ecto-nucleotidases as a circulatory 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 periods 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 recognition and effector function of T, B, and NK cells (27). Alternatively, 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, eicosanoids, EDRF/NO and ecto-ADPase, have important biological properties which merit consideration for therapeutic intervention. Whereas aspirin treatment controls the prothrombotic action of thromboxane, it also prevents formation of antithrombotic 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 hemoglobin 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 biology (3): ADPase/CD39 is an effective physiological and constitutively expressed endothelial cell inhibitor of platelet reactivity. We suggest that ADPase/CD39 offers new opportunities for antithrombotic therapeusis.

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