Suppression of ATP Diphosphohydrolase/CD39 in Human Vascular Endothelial Cells†

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ABSTRACT: Vascular ATP diphosphohydrolase/CD39 is an endothelial cell membrane protein with both ecto-ATPase and ecto-ADPase activities. Suppression of constitutive CD39 expression may result in elevated concentrations of ATP and ADP at the vascular interface that could predispose to thrombosis and inflammation. To study the effects of suppression of CD39 synthesis, stable 25-base antisense chimeric oligonucleotides targeting sequences at the 5′ region of CD39 were designed. Transfection of these stable oligomers into cultured human endothelial cells resulted in dramatic decreases in levels of CD39 mRNA transcripts. Following transfection with antisense oligonucleotides, total ADPase activity fell from 26.0 ± 3.1 in control cultures to 9.5 ± 3.4 nmol of P_i min^{-1} (mg of protein)$^{-1}$ ($p < 0.005$); suppression of CD39 protein expression was also observed by Western blotting. Decreases in ATP diphosphohydrolase activity were associated with increases in concentrations of extracellular purine nucleotides released following stimulation of endothelial cells. Rates of initial hydrolysis of extracellular ATP released from purinergic agonist-stimulated endothelial cells decreased from 17.9 ± 5.0 to 4.8 ± 0.5 pmol min^{-1} per 10^6 cells ($p < 0.005$) in antisense transfected cells. Therefore, CD39 regulates extracellular ATP concentrations and may be an important modulator of purinergic receptor activity in vascular endothelial cells.

Adenine nucleotides are released from activated endothelium and secreted in high concentrations by platelets following their stimulation with exogenous ADP, collagen, thrombin, or activated complement components (*1*, *2*). Purinergic mediators may be responsible for recruitment with sequestration of activated platelets in the microvasculature and could also play a role in endothelial cell (EC)[1] activation responses (*3*, *4*). The modulation of vascular inflammation may be relevant to the pathogenesis of transplant rejection, shock, ischemia, and malignancy (*4*−*7*).

Loss of antithrombotic functions following EC activation may be applicable to the progression of both vasculitis and xenograft rejection (*6*, *8*−*13*). One such regulated vascular thromboregulator is the ectoenzyme ATP diphosphohydrolase (ATPDase; EC 3.6.1.5 or CD39), previously designated as apyrase, ecto-ATPase, or ecto-ADPase (*11*, *14*). CD39 may be a major vascular protective mechanism that modulates blood fluidity and inflammatory responses (*15*, *16*). CD39 rapidly metabolizes both extracellular ATP and ADP to AMP, which is then converted by 5′-nucleotidase to adenosine (*1*, *2*, *17*, *18*). ADP interacts with purinergic type 2 (P2) receptors and is a powerful agonist for platelet recruitment, adhesion, and aggregation; in contrast, adenosine may be an antagonist of these processes (*19*). ATP also interacts with P2 receptors, including P2X7 receptors that induce pore formation in cell membranes (*20*, *21*), and promotes IL-1 release from LPS-stimulated macrophages (*22*) and EC.[2] Moreover, both ATP and ADP may activate neutrophils (*15*) and trigger nitric oxide (NO) release from EC (*23*, *24*). Therefore, by potentially altering levels of purinergic mediators, ATPDase activity may be critical for the regulation of platelet thrombosis and blood fluidity during hemostasis or inflammation (*16*, *25*, *26*).

Antisense oligonucleotides have been used to target mRNA in a sequence-specific manner and thereby inhibit the expression of the specific protein encoded (*27*). Proposed mechanisms of action include inhibition of the following: RNA synthesis, RNA splicing, mRNA export, binding of initiation factors, or assembly of ribosome subunits and their migration (*27*). Antisense oligonucleotides have evolving potential in the treatment of diseases and may be important complementary agents for determination of specific gene functions (*28*).

Having previously identified the ATPDase as CD39 (*25*), we have now examined specific inhibitory reagents to

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[1] Abbreviations: EC, endothelial cell; ATPDase, ATP diphosphohydrolase; NO, nitric oxide; HUVEC, human umbilical vein endothelial cells; mAb, monoclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; P_i, inorganic phosphate.

[2] M. Imai and S. C. Robson, unpublished results.

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Table 1: Antisense Oligonucleotide Target Site of Human CD39/ATPDase mRNA and CD39L1 mRNA[a]

Human CD39	mRNA	1 gaaagaggag gaaaacaaaa gctgctactt **atg**gaagata caaaggagtc taacgtgaag
Human CD39L1	mRNA	1 c tcccgcgcgc ccgcccgccc **atg**gccggga aggtgcggtc actgctgcc
Human CD39	mRNA	51 gccgctgctg acattttgct ccaagaatat cctagccatc cttggcttct cctctatcat˘
Human CD39L1	mRNA	41 ctggccgccg cgggcctcgc cggcctccta ctgctgtgcg tccccacccg cgacgtccgg˘

[a] Nucleotide sequences for human ATPDase cDNA and CD39L1 cDNA obtained with GenBank accession number U87967 (*23*) or U91510 (*27*). The location where antisense oligonucleotides hybridize is underlined. Translation initiation sites are shown in bold and are likewise aligned.

confirm unique biological effects of this vascular ectoenzyme. As there are currently no specific biochemical inhibitors for ATPDase, we have generated CD39 antisense oligonucleotides, complementary to a sequence that includes the translation start site. Suppression of ATPDase activity was associated with substantive changes in levels of extracellular ATP following EC activation in vitro. Putative alterations in purinergic signaling within the vasculature therefore could have biological consequences for both blood flow and fluidity.

EXPERIMENTAL PROCEDURES

Selection of the Target mRNA Sequence. A specific second generation chimeric oligonucleotide 5′-CTC CTT TGT ATC TTC CAT AAG TAG C-3′ and a scrambled control oligonucleotide 5′-TTC GTA TCT TCG CTA GCT TAT ACA C-3′ were designed and purchased from Oligos Etc./Oligo Therapeutics, Inc. (Wilsonville, OR). All studied oligonucleotides have four components: an RNase H activating region, a complementarity zone, and 5′ and 3′ ends. Oligomer design was optimized for thermostability, minimum self-complementarity, and dimer formation. Sequences tested included the initiation start sequence (ATG) and were designed so as not to interact with CD39L1 (*29*). The target site of the antisense oligonucleotide, with the comparable regions of CD39L1, are indicated in Table 1.

Cell Culture and Use of Oligonucleotides. Human umbilical vein endothelial cells (HUVEC) were provided by Dr. B. M. Ewenstein (Brigham and Women's Hospital, Boston, MA) and were used at the third passage. HUVEC were grown to 60−80% confluence on 6 or 10 cm culture plates (Nalge Nunc International, Naperville, IL) with gelatin coated (Sigma, St. Louis, MO) with medium 199 (Bio Whittaker, Walkersville, MD) supplemented with 20% fetal bovine serum (Atranta Biologicals, Norcross, GA), 50 μg/mL epidermal mitogen (Biomedical Technologies Inc., Stoughton, MA), 25 units/mL heparin (Sigma), 50 units/mL penicillin G sodium (GIBCO BRL Life Technologies, Inc., Grand Island, NY), 50 μg/mL streptomycin sulfate (GIBCO BRL), and 2 mM L-glutamine (GIBCO BRL). HUVEC were washed twice with Opti-MEM (GIBCO BRL) prewarmed to 37 °C. Opti-MEM containing 6 μM Lipofectin reagent (GIBCO BRL), 1:1 (w/w) liposome formulation of the cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), and dioleoylphosphotidylethanolamine (DOPE) in membrane-filtered water were preincubated at room temperature for 30−45 min and then added to the HUVEC. Oligonucleotides at a final concentration of 4 μM were incubated with HUVEC for 4 h at 37 °C. Medium was removed, and the cells were washed gently with Opti-MEM and replaced with the complete growth medium. Cells were then cultured for a further 44 h at 37 °C and assayed.

In parallel, COS-7 cells were cultured and transfected with antisense/scrambled control oligonucleotides in the identical manner, 24 h following initial exposure to pCRII or pCRII-CD39 cDNA (*25*).

Localization of Antisense Oligonucleotides. HUVEC were grown on a human fibronectin-coated two-well culture slide (Becton Dickinson Labware, Franklin Lakes, NJ) and treated with 3′ FITC-labeled oligonucleotides (Oligos Etc. Inc.), as described above. After 44 h, cells were washed with DMEM (GIBCO BRL) for 1 min and fixed with 4% paraformaldehyde in DMEM for 5 min at room temperature. After fixation, cells were washed with DMEM for 2 min and dehydrated in a graded series of alcohol (70%−100%) for 1 min each. The subcellular localization of the FITC-labeled oligonucleotides was determined, using a Olympus BX40 fluorescent microscope (Tokyo, Japan).

Intracellular Preparation of RNA and Northern Blotting. Total RNA was isolated from cells by the method of Chomczynski and Sacchi (*30*). RNA samples (20 μg per well) were separated on 1.0% agarose gel containing 10% formaldehyde and then transferred to a nylon membrane (Hybond-N+; Amersham Life Science Inc., Arlington Heights, IL). Integrity, consistency of loading, and transfer of RNA were verified by ethidium bromide staining of ribosomal RNA and by analysis of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. CD39 cDNA (1714 base pair fragment) was used as a probe and GAPDH was used as an internal control probe (*25*). These were labeled with [α-^{32}P]dATP using the Ready-To-Go labeling kit (Pharmacia Biotec Inc., Uppsala, Sweden). Prehybridization, hybridization, and washes of the membrane were carried out according to the rapid hybridization protocol from Stratagene (La Jolla, CA). Final washes were at 55 °C in 0.5 × sodium saline citrate/0.1% SDS for 30 min. The blots were exposed to Kodak Biomax MR film (Eastman Kodak Co.) with intensifying screens for 1 day at −80 °C.

RNA Stability. HUVEC were incubated with 0.25 μg/mL α-amanitin (Boehringer Mannheim GmbH, Germany) for 0, 1, 2, 8, 16, 24, and 48 h. RNA was isolated and analyzed by Northern hybridization with CD39 and GAPDH cDNA as described earlier (*25*).

Cell Lysate Preparation and Western Blotting. After a 44 h incubation (and following the 4 h transfection), EC were washed twice with 20 mM Tris−saline buffer, pH 8.0 at 4 °C on ice, harvested by scraping in Tris−saline containing 0.04 TIU/mL aprotinin (Sigma), and centrifuged at 800*g* for 15 min at 4 °C. Cells were resuspended in Tris−saline, 0.04 TIU/mL aprotinin, 1% Nonident P 40 (Fluka, Ronkonkoma, NY), and 0.1 mM phenylmethanesulfonyl fluoride. This mixture was incubated for 20 min on ice and centrifuged at 16000*g* for 20 min at 4 °C. Supernatants containing cell lysate were used for Western blot analysis and ATPDase

activity assay. Protein concentrations were measured using Bio-Rad Dc protein assay (Bio-Rad Laboratories, Hercules, CA) and read at 750 nm on a microplate spectrophotometer (EL 340; Bio-Tek Instruments, Inc., Winooski, VT).

Proteins (10 μg per lane) were fractionated on a 10% SDS−polyacrylamide gel under nonreducing conditions according to Laemmli (*31*), transferred to polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA) by semidry electroblotting, and then probed with commercial monoclonal antibodies (mAb) to CD39 (Accurate, Westbury, NY). Bands were visualized using horseradish peroxidase-conjugated polyclonal goat anti-mouse IgG (Pierce, Rockford, IL) and Western Blot Chemiluminescence Reagent Plus (NEN Life Science Products, Boston, MA) according to the manufacturer's instructions.

ATPDase Activity Assay. Ca^{2+}, Mg^{2+}-dependent ATPDase activity was determined by measuring inorganic phosphate (P_i) release from ATP or ADP in the presence of 5 mM tetramisole (Sigma) to eliminate effects of contaminating alkaline phosphatases. Enzyme activity was determined at 37 °C in 1 mL of 5 mM $CaCl_2$, 200 μM substrate (ATP or ADP), and 50 mM Tris, pH 8.0, as previously described (*32*). Reactions were stopped by addition of 0.25 mL of malachite green reagent, and inorganic phosphate (P_i) content was estimated according to Baykov (*33*). One unit of ATPDase activity corresponds to the release of 1 μmol of P_i/min at 37 °C. Absorbance was read at 610 nm on a microplate spectrophotometer. Experiments were repeated with different HUVEC cultures in all cases.

Measurement of ATP Release. Transfected or control HUVEC were incubated with DMEM supplemented with 15% fetal bovine serum, 50 units/mL penicillin G sodium, 50 μg/mL streptomycin sulfate, 2 mM L-glutamine, and 100 μM adenosine (Adenocard, Fujisawa, Japan) for 44 h after transfection. At the end of the incubation, the culture medium was changed to DMEM with 10 μM ADP (Sigma) to stimulate HUVEC, and samples of conditioned medium were collected at predetermined intervals (0−60 min) and then reconstituted in EDTA (final concentration 2.6 mM).

ATP concentrations were assayed by modification of the method of McCall (*34*) using a MicroLumat LB96P luminometer (EG&G, Berthold, Germany). In this procedure, ATP is detected by the specific enzymatic reaction of firefly luciferase with luciferin (*35*). Briefly, 20 μL of samples or standards, with 80 μL of 25 mM diglycin (GLY-GLY SigmaUltra, Sigma), 10 mM $MgSO_4$, and 100 ng of luciferase (Sigma), was added to each well of a 96-well immunofluor plate. In the luminometer, 20 μL of 0.2 μM luciferin (Molecular Probes, Inc., Eugene, OR) was added to each well via an automated dispenser, and luminescence was monitored over a 10 s period; all measurements were performed at room temperature. The responses in a given sample or standard were integrated and averaged for several determinations. Data are expressed as micromolar ATP derived from standards examined under the same conditions and plotted over the identical time period examined. Corrected ATP concentrations were then calculated by subtracting any contaminants of ATP in the commercial proportions of ADP. Total ATP released by EC was estimated by measurements of the relevant area under the curve of kinetic plots, and the rates of the initial hydrolysis of ATP were directly calculated.

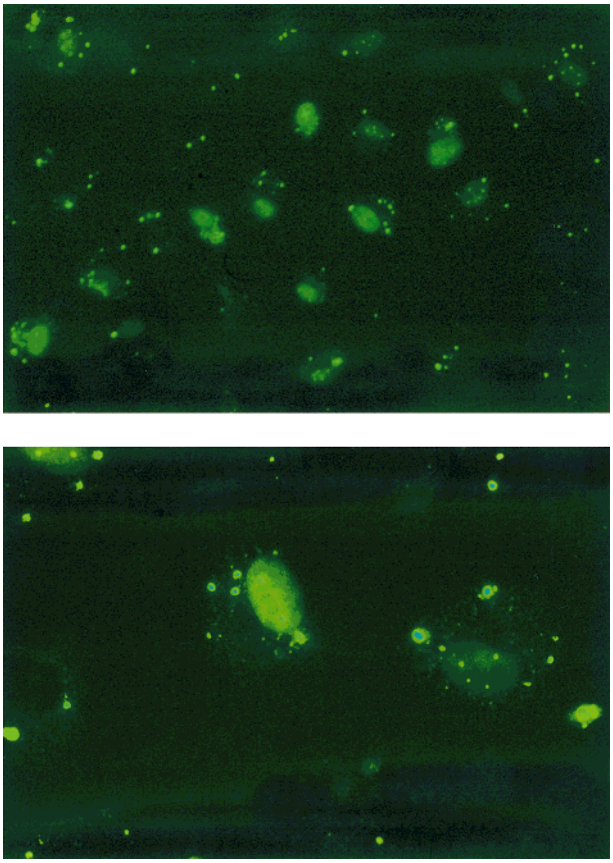

FIGURE 1: Intracellular localization of FITC-labeled antisense oligonucleotides. HUVEC were incubated with 4 μM FITC-labeled oligonucleotides and 6 μM LIPOFECTIN reagent for 4 h at 37 °C and then observed 48 h later by fluorescence microscopy (see text). (A, top) Nuclear staining was observed in 60% of the cells; all show more peripheral cytoplasmic staining (40× magnification; reproduced at 65% of original size). (B, bottom) FITC-labeled oligonucleotides were associated with intracytoplasmic organelles compatible with lysosomes (100× magnification; reproduced at 65% of original size).

Statistical Analysis. The statistical analysis was performed by a two-tailed paired *t* test for comparisons with the control group (Microsoft Excel 5; Microsoft). Differences were considered significant where $p < 0.05$.

RESULTS

Intracellular Localization of the Antisense Oligonucleotides. To evaluate their intracellular penetration and distribution, oligonucleotides were labeled with FITC, and transfected cells were then analyzed by fluorescent microscopy. Cells were incubated with 4 μM FITC-labeled oligonucleotides with Lipofectin and exhibited bright nuclear fluorescence centrally with some bright peripheral cytoplasmic punctuate staining (Figure 1). Over 60% of the cells exhibited nuclear fluorescence; those with cytoplasmic staining approached 100% (Figure 1A). Examination of the cells at higher magnification demonstrated that the cytoplasmic localized FITC-labeled oligonucleotides were associated with organelles suggestive of lysosomal structures, in keeping with previously published data (*36*, *37*) (Figure 1B).

CD39 mRNA Stability. We evaluated the stability of EC CD39 mRNA following suppression of gene transcription with the RNA−polymerase II inhibitor α-amanitin (0.25 μg/mL). RNA was isolated from cells at 0, 1, 2, 8, 16, 24, and

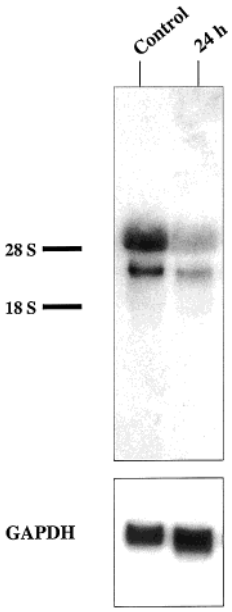

FIGURE 2: CD39 mRNA stability. HUVEC were treated with 0.25 μg/mL α-amanitin for 0 and 24 h. Northern analysis indicated that corrected CD39 mRNA transcript levels had decreased by 93% at 24 h.

48 h and was analyzed by Northern hybridization with CD39 cDNA. Band intensities for the initial CD39 transcripts over 16 h were comparable to baseline when corrected for GAPDH RNA (data not shown). However, Northern blotting confirmed major decreases in corrected CD39 mRNA levels at 24 h (Figure 2). Incubation of cells with α-amanitin for 48 h resulted in 70% loss of cell viability (determined by trypan blue exclusion) that precluded further analysis.

Effect of the Antisense Oligonucleotides on CD39 mRNA Expression. To assess whether oligonucleotides were able to modify levels of CD39 RNA, we performed Northern blotting after transfection using CD39 cDNA (*25*) as the probe. Antisense oligonucleotides specifically inhibited transcription of CD39 mRNA, when compared with scrambled oligonucleotides and control liposomes at 48 h posttransfection (Figure 3). We detected three different mRNA transcripts specific for CD39 as previously described (*25*); all bands were decreased by the specific antisense oligonucleotide treatments.

Effects of the Antisense Oligonucleotides on CD39 Protein Expression. To determine the effect of the decreased RNA levels on CD39 translation, we then studied protein expression by Western blotting in parallel with the Northern analyses. Representative cultures of HUVEC treated with 4 μM ATPDase/CD39 antisense oligonucleotides had decreases in immunoreactive intact CD39 (to 66%), when compared by direct densitometric analysis with control HUVEC exposed to scrambled oligonucleotides (103%) or control liposomes (100%); all cultures were studied at 48 h after transfection (Figure 4). No alterations in the ratio of intact to the 56 kDa proteolytic isoform (*11*) were observed in more exposed autoradiographs (data not shown).

Effect of the Antisense Oligonucleotides on ATPDase Activity. HUVEC and COS-7 cell lysate preparations were incubated with exogenous ADP or ATP to determine ATPDase activity. Specific ADPase activity for HUVEC was 9.5 ± 3.4 nmol of P_i min^{-1} (mg of protein)$^{-1}$ (mean ± SD;

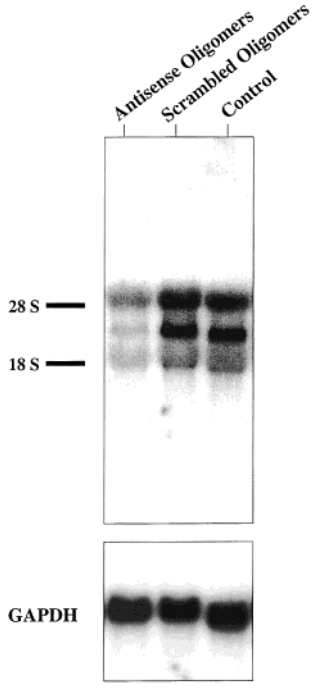

FIGURE 3: Northern analysis. Total RNA (20 μg/well) from the transfected and control cells was hybridized with CD39 cDNA with GAPDH cDNA used as an internal control (*25*). Transfection with antisense oligonucleotides resulted in substantive decreases in CD39 mRNA transcripts.

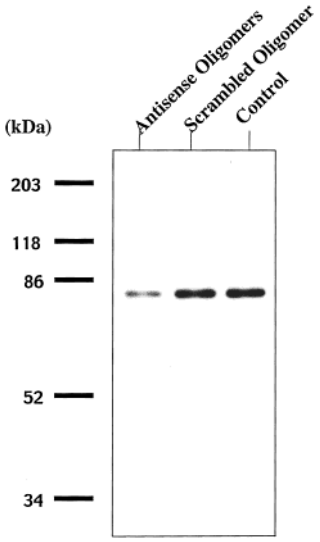

FIGURE 4: Analysis of CD39 expression by Western blotting. Western blotting of transfected HUVEC lysates (10 μg/well) was done using anti-human CD39 mAb (see Experimental Procedures). Antisense oligonucleotide-transfected HUVEC had some decrease in CD39 protein levels when compared to control cultures.

$n = 6$) for HUVEC transfected with antisense oligomers, 22.6 ± 2.1 nmol of P_i min^{-1} (mg of protein)$^{-1}$ with scrambled control oligomers, and 26.0 ± 3.1 nmol of P_i min^{-1} (mg of protein)$^{-1}$ for control liposomes. ATPase activity was 15.1 ± 3.4 nmol of P_i min^{-1} (mg of protein)$^{-1}$ with antisense oligomers, 29.1 ± 1.2 nmol of P_i min^{-1} (mg of protein)$^{-1}$ with scrambled control oligomers, and 28.2 ± 4.6 nmol of P_i min^{-1} (mg of protein)$^{-1}$ for control liposomes (Figure 5). Normalized ADPase activity was significantly inhibited (to 42%) by antisense oligomers when compared with EC incubated with scrambled oligomers ($p < 0.005$; n

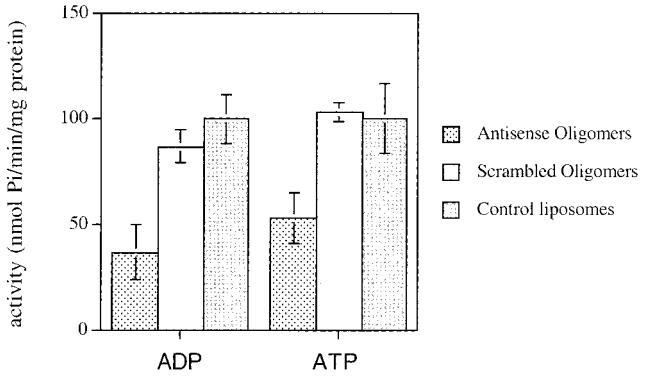

FIGURE 5: ATPDase activity measurement. HUVEC were incubated with or without 4 μM oligonucleotides or no oligonucleotide with 6 μM Lipofectin reagent for 4 h at 37 °C. ATPDase activity was then determined 44 h later. Results are expressed as the mean ± SD of six experiments performed with different batches of HUVEC. Each sample had ATPDase activity analyzed in duplicate. ATPase and ADPase activates in the antisense-treated cell cultures were statistically different from the control groups ($p < 0.005$, by paired *t* test).

$= 6$) or to 36% of control liposomes ($p < 0.005$; $n = 6$). Antisense oligomers inhibited ATPase activity to 51% of that for scrambled control oligomers ($p < 0.005$; $n = 6$) and to 53% of control levels ($p < 0.005$; $n = 6$).

Suppression of ATPDase activity was not due to a toxic effect of the treatment as cell viability determined by trypan blue exclusion remained over 90%. Total cell protein contents from antisense oligonucleotide-transfected HUVEC (17.6 ± 2.8 mg/10^6 cells, mean ± SD; $n = 6$), scrambled control oligonucleotide-transfected HUVEC (17.7 ± 1.8), and control cells (17.9 ± 5.7) were also comparable (data not shown).

In parallel, ATPDase activity was assayed in COS-7 cells, transfected sequentially with CD39 cDNA and oligonucleotides. Antisense oligonucleotides decreased ADPase levels to 15% and ATPase levels to approximately 5% of control cells transfected with CD39 cDNA (data not shown).

Effect of the Antisense Oligonucleotides on ATP Release by Stimulated HUVEC. We evaluated the effects of suppression of ATPDase activity by antisense oligonucleotides on ATP release from ADP-stimulated HUVEC. Rapid induction of ATP release by purinergic-stimulated HUVEC was observed. Peak concentrations of extracellular ATP were comparable in the control and oligonucleotide-treated HUVEC cultures. Antisense oligonucleotide treatment also boosted area under curve estimates of ATP release by 194% (over first 15 min), when compared to controls. Rapid degradation of ATP was observed in minutes in control HUVEC cultures. Treatment with the antisense oligonucleotide prolonged elevated ATP extracellular concentrations (Figure 6). Initial elimination rates of extracellular ATP by hydrolysis were decreased by antisense oligonucleotides, in keeping with the suppression of CD39 expression. The rate of ATP hydrolysis by EC following transfection with antisense oligonucleotides was 4.8 ± 0.5 pmol/min per 10^6 cells (mean ± SD; $n = 9$); using scrambled oligomers the rate was 19.6 ± 0.1 pmol/min per 10^6 cells and for liposome-treated controls 17.9 ± 5.0 pmol/min per 10^6 cells.

DISCUSSION

ATPDase/CD39 represents a pivotal member of the rapidly expanding GDA 1/CD39 NTPase family (*29*, *38*, *39*) and

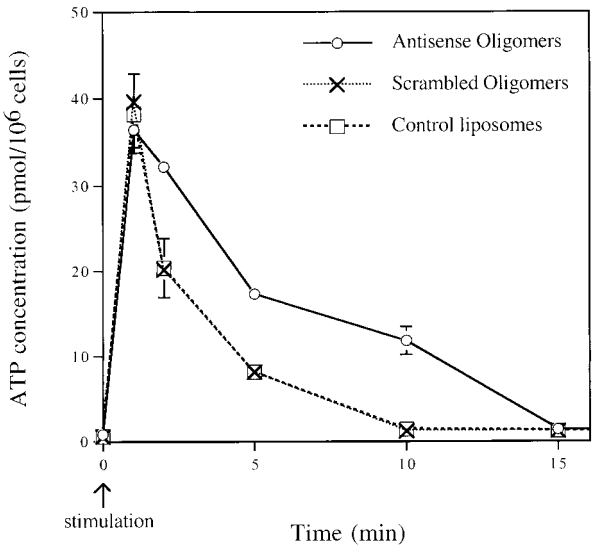

FIGURE 6: ATP degradation by exogenous ADP-stimulated HUVEC. HUVEC were transfected with oligonucleotides and 48 h later stimulated with 10 μM ADP in DMEM. Supernatants were collected and extracellular ATP concentrations determined. Suppression of ATPDase activity was associated with decreased levels of hydrolysis and, consequently, increased concentrations of ATP from 2 to 15 min, when compared to the control cultures.

may play an important role in the modulation of both platelet (*40*) and EC activation responses (*42*). However, there are no specific inhibitors to CD39, and other ectonucleotidase homologues have recently been identified. Both of these factors complicate delineation of unique biochemical functions and pathophysiological importance of the individual ectonucleotidases in various cell types.

Substantial suppression of ATPDase/CD39 activity was achieved in both EC and transfected COS-7 cells following treatment with antisense oligomers *in vitro*. Such decreases in levels of expressed ATPDase activity were demonstrated to have potential biological effects with respect to alterations in concentration of extracellular adenine nucleotides in vitro. Although initial levels of ATP secreted by activated EC were comparable in both control and treated cells, substantive delays in hydrolysis of extracellular ATP were observed following the use of antisense oligomers to CD39, in keeping with the suppression of ATPDase expression. Comparable changes in the purinergic signaling environment could have major implications for the evolution of vascular activation responses *in vivo*.

The mechanisms of purinergic signaling and their importance in modulating hemostasis, vascular inflammation, and thrombotic reactions remain unclear (*20*). Several processes of vascular injury with organ reperfusion or those associated with graft rejection (*4*, *7*) result in the release of ATP and ADP from platelets and endothelium (*1*, *2*).[3] These mediators modulate both platelet adhesion to injured subendothelium and platelet recruitment to rapidly form occlusive thrombi (*40*), in keeping with the time frame of our experiments. ATP and ADP may also trigger NO secretion from EC by stimulation of purinergic type P2Y receptors (*23*, *24*). This latter action is believed to limit the extent of intravascular platelet aggregation and to help localize platelet microthrombus formation to areas of vascular damage (*15*, *41*). Recently,

[3] M. Imai, R. N. Pierson, and S. C. Robson, unpublished results.

we have demonstrated that both ATP and ADP activate transcription factor NF-κB and can induce HUVEC apoptosis potentially by activation of P2X7 receptors (*42*). Purinergic signaling can also influence vasomotor responses, platelet activation, and inflammatory processes as well as cardiac function (*20*−*22*, *43*, *44*). Adenosine derived from adenine nucleotides, through the action of ectonucleotidases, will interact with specific P1 receptors that may often exert effects opposite to those elicited by P2 receptors (*45*). However, this picture is further complicated by purinergic receptor desensitization phenomena; in this specific manner, nonhydrolyzed adenine nucleotides at the vascular interface may also limit ADP-mediated reactivity of platelets (*44*, *46*, *47*), as we have observed in cd39 deficient mice (*50*).

In the vasculature, the actions of extracellular nucleotides may be limited by their degradation by ectonucleotidases, phosphatases, and pyrophosphatases (with uptake of adenosine) (*48*). Cellular consequences of CD39 deficiency could be associated with the failure of adenosine cycling, dysregulation of ecto-kinase substrate concentrations, and disordered purinergic signaling (*17*). Deletion of cd39 in mutant mice results in platelet P2Y1 desensitization with aberrant EC expression of tissue factor (*50*). Exogenous adenosine was added to EC cultures to provide adequate levels of intracellular ATP for the stimulation experiments. Identical ATP release profiles were observed in all cell cultures, irrespective of levels of CD39 expression and indicative of adequate ATP storage despite antisense treatments. The major differences observed in antisense oligomer treated EC were that high levels of released ATP persisted, in that the kinetics of elimination were retarded by suppression of CD39 expression. Associated delays in extracellular adenosine formation and uptake would likely ensue. Higher ATP concentrations could result in higher levels of purinergic stimulation with promotion of NF-κB-mediated pathways (*42*), in addition to NO release (*23*, *24*) or even in desensitization responses (*50*).

Our data derived from the mRNA stability experiments suggest that CD39 mRNA appears to be stable for up to 16 h but that by 24 h most specific transcripts have been degraded. The manifestations of antisense oligonucleotide administration with respect to CD39 mRNA levels were tested when the anticipated effects would be maximal (Figure 3). Substantive decreases in the levels of specific RNA transcripts were therefore observed when tested at 48 h. However, the effects of the antisense oligonucleotide on CD39 protein expression, as determined by qualitative Western blotting, were less dramatic with approximately 40% apparent decreases observed (Figure 4). Previous work by us and others has demonstrated that the presence of immunoreactive CD39 antigen alone does not correlate with functional ATPDase activity; the formation of multimers (*49*) or oxidative inactivation are added factors that influence enzymatic activity (*11*). Experiments consistently demonstrated that the bulk of EC ecto-ADPase activity could be inhibited by antisense oligomers directed at CD39 (Figure 5). Less substantive and relative decreases in ecto-ATPase levels may be in keeping with prior observations of the coexpression of homologues of CD39 such as CD39L1 on EC (*38*, *50*). The modest decrease in CD39 immunoreactivity also did not directly correlate with the substantial simultaneous decreases in specific expression of ATPDase activity and suggested that the persistent expression of immunoreactive CD39 protein did not indicate the full potential for biochemical activity. In addition, no increase in proteolytic degradation to the 56 kDa isoform (*49*) was observed. In COS-7 cells, suppression of new CD39 synthesis by antisense oligonucleotides was far more efficient and in keeping with the contribution of newly synthesized CD39 to the cellular ATPDase activity (data not shown). All of these findings are consistent with our published observations that decreases in ATPDase activity in vascular EC may occur without proteolytic degradation or immediate loss from the plasma membrane and suggest that functional inactivation of CD39 may occur without loss of cellular immunoreactivity (*11*).

We have already established that vascular ATPDase/CD39 expression may be rapidly decreased by reperfusion injury (*8*), oxidant stress (*12*, *25*), or cytokine-mediated EC activation responses (*11*). Our data suggest that loss of ATPDase activity, following suppression of CD39 synthesis, may result in decreases in extracellular adenine nucleotide hydrolysis. In the current study, we show that substantial loss of specific ATPDase activity results in persistent elevations in concentrations of ATP in the extracellular environment. Currently, it seems reasonable to speculate that CD39 and purinergic mediators act in concert to modulate blood fluidity and platelet activation. Our *in vitro* data provide a further rationale on how the loss of vascular ATPDase may modulate progression of vascular injury (*11*).

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