Analysis of CD39/ATP Diphosphohydrolase (ATPDase) Expression in Endothelial Cells, Platelets and Leukocytes

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Summary

Purinergic signaling may influence hemostasis, inflammatory responses and apoptosis. Therefore, hydrolysis of extracellular ATP and ADP by the ATP diphosphohydrolase (ATPDase) could regulate these processes. We have previously demonstrated the identity between the vascular ATPDase and CD39. Here we show that levels of CD39 expression correlate with ATPDase activity in human endothelial cells (EC), platelets and selected monocyte, NK, and megakaryocyte cell lines. Western blotting revealed one to three isoforms of CD39/ATPase; mobility variations of major protein resulted from post-translational modifications. Northern blotting and primer extension indicated two major mRNA transcripts and one transcription start point, respectively. In addition, mRNAs specific for purinergic P2 receptors were detected in all of the investigated cells, suggesting that the co-expressed CD39/ATPase may regulate purinergic signaling. Thrombotic and inflammatory responses may be modulated by the expression of CD39/ATPase.

Introduction

ATP diphosphohydrolase (ATPDase, EC 3.6.1.5) is a plasma membrane-bound enzyme that plays a dominant role in the hydrolysis of extracellular tri- and/or diphosphate nucleotides (1). Nucleotides present in circulation may be released from activated cells or damaged tissues and are involved in platelet aggregation, cardiac contractility, vascular tone regulation and neurotransmission (2). In addition, extracellular ATP has been recognized as a mediator of many immune and non-immune cell functions. Purinergic stimulation may influence cell apoptosis and necrosis (3), DNA synthesis (4), histamine secretion (5), and secretion of granules from neutrophils or monocytes (6, 7). ATP inhibits cytotoxicity mediated by macrophages (9) and NK cells (10, 11), but also represents an effector molecule in cytotoxic T lymphocytes-mediated lysis (12).

Adenine nucleotides exert variety of effects on different cell types through interactions with specific P2 receptors. These receptors have been recently classified into a G-protein-coupled family of P2Y receptors and a ligand-gated family of P2X receptors (13). A member of the P2X receptor family, P2X7 (previously classified as P2Z), has been implicated in ATP-induced permeabilization of cell membrane resulting in cell apoptosis (14).

Vascular ATPDase inhibits platelet aggregation by the hydrolysis of the potent platelet activator, ADP, to AMP and thus acts as a dominant thromboregulatory factor. Recently we and others have demonstrated the common identity between the vascular ATPDase and CD39 using immunological and functional analyses (15-17). CD39 had been previously shown to be a B lymphocyte activation marker and to be expressed on the surface of NK cells, cytotoxic T lymphocytes and monocytes (18). Several other reports have described ATPDase activity on blood cells (19, 20). No studies have related ATPDase activity to CD39 expression. The recognition of vascular ATPDase as CD39 led us to the assumption that CD39 expressed on blood cells also had identity to ATPDase.

Monocytes, NK cells and platelets are involved in inflammation and thrombosis and are in contact with the blood vessel wall. Thus, ATPDase expressed on endothelium and these blood cells could modulate the effects of ATP and ADP within the vasculature and at sites of inflammation.

In this report, we examine the forms of CD39 expression by human endothelial cells (EC), platelets and selected monocyte, NK and megakaryocyte cell lines and correlate this with ATPDase activity. In addition, we confirm that CD39/ATPase is co-expressed with P2 receptors and indicate a potential role of the enzyme in modulating purinergic receptor signaling and apoptosis.

Materials and Methods

Cell Culture

Human Mø01 cells (megakaryoblastic cell line) were grown in RPMI 1640 medium supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), glutamine (2 mM), and 10% heat inactivated fetal calf serum (FCS). Supplemented Dulbecco's modified Eagle's minimal essential medium was used for cultivating human promonocytic lymphoma cell line U-937 (21). The above cell lines were obtained from the American Type Culture Collection (Rockville, MD). NK92 cell line (22) was maintained in MyeloCult H1500 medium (StemCell Technologies Inc, Vancouver, Canada) in the presence of IL-2 (500 U/ml, R&D Systems, Inc., Minneapolis, MN). Human umbilical vein EC (HUVEC) were provided by Dr. B. Ewenstein, Brigham and Women's Hospital, Boston, MA. HUVEC were cultured in M199 (BioWhittaker, Walkersville, MD) supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), glutamine (2 mM), 20% FCS, heparin (100 μg/ml) and endothelial mitogen (50 μg/ml) (Biomedical Technologies Inc., Stoughton, MA).
ATPDase Biochemical Activity Assay

Membrane-bound ATPDase ecto-enzymatic activity was determined by measuring the amount of liberated inorganic, radioabeled phosphate hydrolyzed from [γ-32P] ATP (NEN Life Science Products, Boston, MA) (23). The reaction mixture added to cells (final volume of 0.2 ml) contained 3 mM ATP and 0.3 μCi of [γ-32P]ATP in 10 mM Hepes, pH 7.4 with 135 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM glucose and 1% bovine serum albumin. After 10 min incubation at 37°C the reaction was terminated by the addition of 0.5 ml of cold 20% (v/v) activated charcoal in 1 M HCl. The samples were cooled on ice for 10 min and then centrifuged at 3200 × g for 4°C for 20 min. Aliquots (0.2 ml) of the supernatants were transferred to scintillation vials containing 2.5 ml Ultima Gold scintillation mixture (Packard, Meriden, CT) and counted in a Liquid Scintillation Analyzer (Packard, Downers Grove, IL) for 32P.

Additionally, intact cells were incubated in 20 mM Tris pH 8.0 containing 5 mM CaCl2, and either 200 μM ADP or 200 μM ATP for 15 min at 37°C, and release of free phosphate was determined with a non-radioactive technique. Five mM tetramisole was added to inhibit alkaline phosphatase. A standard curve was constructed using 0-20 μM KH2PO4. Malachite green was added to stop the reaction and absorbance at 610 nm was measured to determine phosphate levels against the standard curve (24).

For both methods, each experiment was repeated at least three times and the mean ± standard deviations were calculated where appropriate.

Cytofluorometric Analysis of the CD39 Expression

HUVEC were harvested by gentle pipetting with Hanks balanced salt solution (GIBCO-BRL, Grand Island, NY) containing 10 mM EDTA. Human blood platelets were prepared by gradient centrifugation of anticoagulated blood in Cellsep solution exactly as recommended by manufacturer (Larex, St. Paul, MN). HUVEC, U937, NK92, Meg01 cells and platelets were washed with buffer (PBS, 5% FCS, 0.02% sodium azide) and incubated with either anti-human CD39 monoclonal antibody (mAb), BU61 (IgG1) (Accurate, Westbury, NY) or an isotype matched control mAb, anti-CD25/ACT-1 (IgG) (Dako, Carpinteria, CA) for 30 min on ice. Cells were washed twice and incubated with anti-mouse IgG conjugated with fluorescein-5-isothiocyanate (FITC) (Sigma, St. Louis, MO) for 30 min on ice. Finally cells were washed twice and analyzed by flow cytometry on a FACScan bench top model using Cellquest II software (Becton Dickinson, San Jose, CA). Data were collected from viable cells only, as determined by propidium iodide uptake.

Cell Lysate Preparation

HUVEC were washed 3 times with Tris-saline buffer, pH 8.0 at 4°C and harvested by scraping in 20 mM Tris, pH 8.0, 50 mM NaCl solution. HUVEC, U937, NK92, Meg01 cells and platelets were lysed in buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.02 KIU/ml aprotinin, 0.5 μg/ml leupeptin and 1% Nonidet P40 (NP40). Cells were centrifuged 10,000 × g for 10 min at 4°C and supernatants containing cell lysates were then used for Western blot analysis and deglycosylation experiments.

Western Blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (25). Cell lysate proteins (20 μg per lane) were separated on 10% polyacrylamide gel under non-reducing conditions, transferred to PVDF membrane (Immobilon P, Millipore, Bedford, MA) by electroblotting and probed with monoclonal antibody to human CD39 (Accurate, Westbury, NY). Bands were visualized using horse radish peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL) and the Enhanced Chemiluminescence assay (Amer sham Life Science Inc., Arlington Heights, IL) according to the manufacturer’s instructions.

RNA Isolation and Northern Analysis

RNA samples from HUVEC, U937, NK92, Meg01 and platelets were isolated according to the method described by Chomczynski and Sacchi (26).

Total RNA from the studied cell types (20 μg per sample) was separated on a 1% formaldehyde-agarose gel and transferred onto nylon membrane (Amersham Life Science Inc, Arlington Heights, IL) by capillary blotting, followed by UV cross-linking. RNA was hybridized with CD39 cDNA (GenBank accession No. S73813). This cDNA was labeled with [α-32P]dATP using Ready-To-Go labeling kit (Pharmacia, Piscataway, NJ). Prehybridization (30 min) and hybridization (1 h) were carried out at 65°C in the Rapid-hyb buffer (Amer sham Life Science Inc., Arlington Heights, IL). Membranes were washed twice for 15 min in 2× sodium saline citrate (SSC)/0.1% SDS at room temperature and once for 30 min in 0.5 × SSC/0.1% SDS at 55°C. The blots were exposed to Kodak Biomax MR film (Eastman Kodak Company, Rochester, NY) with intensifying screens at -80°C for 24 h.

RT-PCR

Total RNA prepared from all the studied cell lines and platelets (5 μg per sample) was annealed with oligo(dT)15 primer. First strand cDNA synthesis was carried out with Superscript Reverse Transcriptase (GIBCO-BRL, Grand Island, NY). The product of this reaction served as a template for PCR amplification. The following primers (Midland Certified Reagent Co., Midland, TX) were used to amplify CD39 cDNA 792-1672 fragment: forward primer: 5'-ATGGCAAGGACTACAATGT3'- and reverse primer: 5'-CACCAGTGGGAGAAAT-3'. For human P2Y1 receptor (GenBank accession No. Z49205) a set of primers was designed (forward primer: 5'-ATCGGCTCTCTGGGCAACA-3' and reverse primer: 5'-CCAAGGGGACACAGAACAT-3') to generate 1039-1538 cDNA fragment. For human P2X4 receptor (GenBank accession No. AF000234) we amplified 151-1131 cDNA fragment with forward primer: 5'-GGATGTTGGCGATTGTGT-3' and reverse primer: 5'-TCCTCCCTCCTCCCTCCTC-3'. For human P2X7 receptor (GenBank accession No. Y09561) 1184-1853 cDNA fragment was amplified with forward primer: 5'-GAATGGGAGCCATTGTTG-3' and reverse primer: 5'-AAAGGTGGGATTACAG-3'. The reactions were carried out in MJ Research Thermal Cycler (Watertown, MA) for 35 cycles (0.5 min at 94°C; 1 min at 53°C for CD39, 56°C for P2Y1, P2X4, and P2X7; 2 min at 72°C) followed by 10 min at 72°C. Amplified PCR products were examined on 1% agarose gel. PCR negative controls contained water instead of cDNA.

Southern Hybridization

PCR products were run on 1.8% Agarose gel in TBA buffer, transferred to a nylon membrane (Hybond+), UV-crosslinked and hybridized with CD39 cDNA as described by Sambrook et al. (27). CD39 probe was labeled with [α-32P]dATP using Strip-EZ DNA kit (Ambion Inc., Austin, TX). Prehybridization (30 min) and hybridization (1 h) were carried out at 65°C in the Rapid-hyb buffer (Amer sham Life Science Inc., Arlington Heights, IL). Membranes were washed twice for 15 min in 2× SSC/0.1% SDS at room temperature and once for 30 min in 0.5 × SSC/0.1% SDS at 55°C. The blots were exposed to Kodak Biomax MR film (Eastman Kodak Company, Rochester, NY) with intensifying screens at -80°C for 20 min.

Primer Extension Analysis

Primer extension analysis was used to determine the location of the 5'-end of the specific CD39 RNA and performed according to the manufacturer's instructions (Promega, Madison, WI). Briefly, an oligonucleotide complementary to CD39 cDNA sequence (antisense primer starting at position 28 in CD39 accession number to H9262) was amplified 151-1131 cDNA fragment with forward primer: 5'-GAATGGGAGCCATTGTTG-3' and reverse primer: 5'-AAAGGTGGGATTACAG-3'. The reactions were carried out in MJ Research Thermal Cycler (Watertown, MA) for 35 cycles (0.5 min at 94°C; 1 min at 53°C for CD39, 56°C for P2Y1, P2X4, and P2X7; 2 min at 72°C) followed by 10 min at 72°C. Amplified PCR products were examined on 1% agarose gel. PCR negative controls contained water instead of cDNA.
analyzed on 8% polyacrylamide gel in Tris-HCl, borate, EDTA (TBE) buffer containing 7M urea and ran at 250 V in TBE buffer. The length of the generated cDNA reflected the number of bases between the primer’s binding site and the transcription start point.

Deglycosylation

Cell lysates from all analyzed cell lines were subjected to deglycosylation using N-glycosidase F (PNGase F). One hundred µg of heat denatured protein (100°C, 5 min) was incubated with 200 mU PNGase in 0.5 M Tris-HCl, pH 8.0, 0.1 M 1, 10-phenantroline, 10% NP40, at 35°C overnight. Samples were then analyzed by Western blotting as described above.

Results

ATPase Biochemical Activity

ATPase activity on intact cells was measured by inorganic phosphate release using two techniques with either [γ-32P] ATP or ATP and ADP as substrates. Values obtained with these methods for the various cell types differed slightly, potentially due to differences in substrate, buffer composition and pH, and method of released phosphate detection. The highest ATPase activity was associated with HUVEC (Fig. 1A and B) and approximately 5 times lower activity was observed for NK92, for both, ATP and ADP (Fig. 1B). ATPase activity on U937, Meg01 and platelets was detectable, but at considerably lower levels.

Immunological Identification of CD39/ATPase on Different Cell Types

CD39/ATPase expression analysis by flow cytometry. Using mAb to human CD39, we performed flow cytometry to evaluate the expression of CD39 on cell surface (Fig. 2). A strong fluorescence shift was observed for HUVEC, U937 and NK92. Meg01 and platelets stained weakly.

Western analysis. HUVEC, NK92, Meg01 and platelet cell lysates were analyzed for presence of CD39/ATPase by Western blotting using mAb to CD39. SDS-PAGE was performed under non-reducing conditions to maintain reactivity with BU61 monoclonal antibody. Consistent differences in CD39/ATPase mobility for analyzed cells were revealed (Fig. 3). Three distinct polypeptide isoforms were noted for HUVEC (approximately 78, 56 and 42 kDa), two for NK92 (~92 and 64 kDa), with predominantly one for U937 (~94 kDa), Meg01 (~73 kDa) and platelets (~78 kDa). The lower bands detected for HUVEC and NK92 were a result of posttranslational modification, due to limited proteolysis (manuscript in preparation). In platelet lysates, a
non-specific band exceeding 200 kDa (recognized by secondary antibody alone) was also observed.

Detection of CD39/ATPDase by RT-PCR Followed by Southern Hybridization

RNA isolated from investigated cells was subjected to RT-PCR. CD39 cDNA fragment of 881 bp was generated for all studied cells (Fig. 4A). To confirm authenticity of these fragments, obtained cDNAs were probed with CD39 cDNA by Southern blotting (Fig. 4B). cDNA fragments from all studied cells hybridized with CD39 probe. The cDNAs fragments were also subjected to restriction digests and the expected pieces of cDNA were observed (data not shown).

CD39/ATPDase mRNA Analysis by Northern Hybridization and Primer Extension Analysis

Differences in CD39/ATPDase mobility detected by Western blotting suggested that variation in the specific mRNAs could be a reason for this observation.

RNA isolated from cells was analyzed by hybridization with CD39 cDNA. Two major mRNA transcripts were visible in all studied cells (Fig. 5). We looked also for differences in transcription start point for various cell types by primer extension analysis (Fig. 6). Radiolabeled primer was hybridized with total RNA and reverse transcriptase reaction was performed. A distinct approximately 86 bp fragment was generated for HUVEC, U937, NK92 and Meg01. This result indicated that there were approximately 86 bases between labeled nucleotide of the primer and the beginning of transcription in all the cells analyzed. The synthesized fragment was about 40 bp longer than the published cDNA sequence.

Deglycosylation

Since variations in SDS-PAGE mobility of CD39/ATPDase from studied cells could not be explained by RNA diversity, we expected that posttranslational modifications might be a possible reason for observed differences. Amino acid sequence analysis of the human CD39/ATPDase revealed 6 possible N-glycosylation sites (35). To investigate diversity in CD39/ATPDase glycosylation, cell lysates from HUVEC, U937, NK92, Meg01 and platelets were digested with PNGase F to remove N-linked saccharides. In a subsequent analysis by Western blotting the monoclonal antibody to human CD39 recognized two
bands of approximately 54 and 35 kDa for HUVEC and one band of approximately 54 kDa for NK92, U937 and platelets (Fig. 7). Although this same 54 kDa product of deglycosylation was visible for Meg01, an additional prominent protein band was slightly bigger (58 kDa).

Detection of Purinergic Receptors by RT-PCR

We assumed that CD39, by hydrolysis of extracellular ATP and ADP, may affect purinergic signaling. We investigated the distribution of mRNA encoding human purinergic receptors, P2Y1, P2X4 and P2X7 in all of the studied cells and platelets. Amplified products of PCR were analyzed by agarose gel electrophoresis (Fig. 8). The generated P2 receptors cDNAs had expected sizes (499, 980, and 669 bp for P2Y1, P2X4, and P2X7, respectively), and their identity were confirmed by restriction enzyme digests (data not shown). In PCR negative controls containing water instead of cDNA, no band was generated. All studied cells and platelets expressed at least two of the investigated purinoceptors.

Discussion

Although CD39 expression had been noted on B- and T-lymphocytes and NK cells, ATPDase activity had not been directly correlated with the presence of CD39 antigen. Recently, we have shown that CD39 derived from HUVEC had all the characteristics of ATPDase (15). On this basis, we assumed that ATPDase activity associated with other blood cells was also the result of CD39 expression. In this report we have examined and contrasted CD39/ATPDase expression and activity in EC, platelets and various human cell lines representing monocytes, NK cells and megakaryocytes.

ATPDase activity was confirmed to be of ecto-enzyme origin since intact cells (and not cell-lysates) were analyzed and an inhibitor of alkaline phosphatase, tetramisole, was present in the assay. Our results indicated that enzymatic activity expressed on HUVEC was markedly higher than on other cells (Fig. 1). This finding confirmed that EC ATPDase is the dominant contributor to the ATP and ADP hydrolysis within the vascular system. ATPDase associated with NK92 was the next most active in degradation of ATP and ADP. Little is known about regulation of CD39/ATPDase expression. We have shown that vascular ATPDase is down-regulated at the protein level by TNFα and oxidants (36). Upregulation of CD39 has been associated with activation of B- or T-lymphocytes, and NK cells in long-term cultures (18). NK92 cells used in our experiments were cultured in the presence of IL-2 required for their growth that could account for their relatively high ATPDase activity.

Immunological identification of CD39/ATPDase on different cell types was performed by cytofluorometric and Western blot analyses. We confirmed that the studied cells expressed protein immunoreactive with anti-human CD39 (Fig. 2). Strong signals were observed for HUVEC, NK92 and U937. Platelets and Meg01 had much lower levels of CD39 expression.

Western blotting also revealed differences in size and degree of CD39/ATPDase expression (Fig. 3). Three or two distinct polypeptide isoforms of CD39 were observed for HUVEC and NK92, respectively. Lower molecular mass bands appear to be proteolytic degradation products (37). One protein band of different size was detected for U937, Meg01 and platelets.

The differences in CD39/ATPDase molecular mass observed by Western blotting could be caused by either mRNA diversity (alternative splicing, differential transcription start or stop sites) or posttranslational modifications such as glycosylation and/or limited proteolysis. To investigate these variations in the protein size, we performed CD39 RNA analysis as well as deglycosylation of CD39 protein expressed on the studied cells.

By RT-PCR, 881 bp cDNA fragments were generated for all studied cells (Fig. 4A) and their identity were confirmed by Southern hybridization with CD39 cDNA (Fig. 4B). With this method we were able to generate cDNA fragments covering whole CD39 mRNA sequence for all studied cells. Northern blotting and primer extension results suggested that there were no differences in CD39/ATPDase mRNA isolated from the analyzed cells. Two major transcripts were visible by Northern hybridization (Fig. 5) in agreement with previously presented results (15). One dominant transcription start point for tested RNAs was observed as analyzed by 5′-primer extension method (Fig. 6). The determined length of the generated CDNA in primer extension experiment suggested that transcription started approximately 86 bases from the labeled primer binding site. This fragment was approximately 40 bp longer than the published sequence of the human CD39 cDNA (35). As CD39 cDNA hybridized with two major mRNAs, the presence of one transcription start point suggested some potential differences in the 3′ region of CD39 mRNA.

Human CD39 amino acid sequence examination has indicated 6 putative N-glycosylation sites (35). The hydrolysis of N-linked oligosaccharides with PNGase F in cell lysates changed the observed electrophoretic pattern (Fig. 7 versus Fig. 3). After deglycosylation, a band of
approximately 54 kDa was detected for HUVEC, NK92, U937 and platelets. Although the same ~54 kDa product of deglycosylation was visible for Meg01, the major protein band was slightly bigger (~58 kDa). Either the deglycosylation reaction of Meg01 synthesized CD39/ATPase protein was incomplete or other posttranslational modifications were responsible for the final size of this ecto-enzyme. For HUVEC additional deglycosylated isofrom of ~35 kDa, originated from the proteolysis of native protein, was observed. These results correspond to other reported data demonstrating similar shift in molecular mass after deglycosylation for bovine aorta ATPase and pig pancreas ATPase after PNGase F digestion (33, 38). It appeared that CD39/ATPase protein core of ~54 kDa was the same for the investigated cells. Our data strongly indicated that variations in CD39/ATPase mobility were caused by posttranslational modifications.

We assume CD39/ATPase function is correlated with its localization and could therefore be influenced by cellular environment. Vascular CD39/ATPase may influence the function of blood cells that interact with EC, e.g., a characterized function of EC CD39/ATPase has been related to the inhibition of ADP induced platelet aggregation and activation (15, 39). CD39 on activated B lymphocytes has been shown to play a role in homotypic cell adhesion (18). The function of CD39/ATPase present on monocytes, NK cells, megakaryocytes, and platelets has not been yet established. It is tempting to speculate that the enzyme can be involved in cell signaling initiated by ATP/ADP directly or indirectly by hydrolysis of agonists for purinergic receptors (article in preparation).

Type-2 purinergic receptors (P2) have been localized on several cell types, e.g., macrophages (41), lymphocytes (42-44), platelets/megakaryocytes (45, 46) and EC (47, 48). Each of the studied cells expressed purinergic receptors (Fig. 8). P2X1 and P2Y1 receptors have been previously described on blood platelets and megakaryoblastic cell line (45, 46, 47). Their involvement in platelet aggregation and activation has been evaluated (49, 50). Our data confirmed the presence of P2Y1 receptors mRNA in both platelets and megakaryocytes. We found mRNA for P2X7 receptors in platelets, megakaryocytes and lymphoid cell lines. To date this receptor was associated only with cells of the immune/inflammatory system and rat hepatocytes (51). P2X7 receptor detection on platelets by RT-PCR is a novel observation. The detection of this receptor in a megakaryocytic cell line strongly confirms the result obtained for platelets. P2X7 receptor has been associated with apoptosis (52), mitogenic stimulation in T cells (53), cytokine release from macrophage and microglial cell lines (54, 55), some types of cytotoxicity (56), and NFκB activation (57). As we have already noted, ATP can initiate EC apoptosis probably mediated by P2X7 receptors with NFκB activation involvement (58).

ATPase expressed by the investigated cells might influence signal transduction initiated by purinergic receptors by hydrolysis of their agonists. We assume that activity and expression of CD39/ATPase in the vasculature and other cells may have profound consequences for modulation of signals triggered by purinergic receptors, including apoptosis, anti-inflammatory and prothrombotic responses.

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