Adherent Leukocytes Prevent Adenosine Formation and Impair Endothelial Barrier Function by Ecto-5′-nucleotidase/CD73-dependent Mechanism*

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Tiina Henttinen, Sirpa Jalkanen, and Gennady G. Yegutkin‡

From the MediCity Laboratory and Department of Medical Microbiology, Turku University and National Public Health Institute, FIN-20520 Turku, Finland

Extracellular purines are important signaling molecules that mediate both inflammatory (ATP, ADP) and anti-inflammatory (adenosine) effects in the vasculature. The duration and magnitude of purinergic signaling is governed by a network of purino-converting ectoenzymes, and endothelial and lymphoid cells are generally characterized by counteracting ATP-inactivating and ATP-regenerating/adenosine-eliminating, phenotypes, respectively. By using cultured human umbilical vein endothelial cells and normal or leukemic lymphocytes as an in vitro model of leukocyte-endothelial interactions, we have identified a link between the adhesion cascade and extracellular purine turnover. Upon adhesion, lymphocytes suppress endothelial purine metabolism via (i) inhibition of ecto-5′-nucleotidase/CD73-mediated AMP hydrolysis, (ii) rapid deamination of the remaining adenosine, and (iii) maintenance of the sustained pericellular ATP level through continuous nucleotide release and phosphotransfer reactions. Compensation of the loss of adenosine promotes vascular barrier function (measured as a paracellular flux of 70 kDa fluorescein isothiocyanate-dextran) and decreases transendothelial leukocyte migration. Together, these data show that adherent lymphocytes attempt to prevent adenosine formation in the endothelial environment that, as a consequence, may impair the vascular barrier function and facilitate the subsequent step of leukocyte transmigration into the tissue. These leukocyte adhesion-mediated shifts in the local nucleotide and nucleoside concentrations represent a previously unrecognized paracrine mechanism affecting the functional state of the targeted vascular endothelium and coordinately regulating lymphocyte trafficking between the blood and tissues.

Lymphocyte trafficking between the blood and tissues is essential for the proper function of the immune system. Present hypotheses suggest a multistep model of the leukocyte extravasation, including transient lymphocyte adhesion to the endothelial lining, rolling of the cell along the endothelium, cellular activation, activation-dependent arrest, and finally, transmigration into the tissue (1). Leukocyte-endothelial interactions are dynamic, involve both cell adhesion through specific receptor-ligand pairs and bidirectional cell signaling, and are affected by soluble mediators, which can modulate adhesive molecules and signaling events in both cell types (2). The role of extracellular purines as important cell modulators has emerged relatively recently. Most models of purinergic signaling depend on functional interactions between distinct processes, including (i) release of endogenous nucleotides through channel-like pathways, secretory exocytosis, or leakage upon cell damage (3, 4); (ii) triggering of signaling events (5–7); and (iii) ectoenzymatic nucleotide inactivation and interconversion (8–10). A number of observations raised the possibility that ATP and/or ADP regulate several inflammatory responses, including recruitment of neutrophils and monocytes to the site of injury, facilitation of leukocyte adhesion to the endothelium, platelet activation and aggregation (6, 11–13), exacerbated skin inflammation (14), and rapid microvesicle shedding of the interleukin-1β from activated monocytes (15). Most of these effects are mediated through the G protein-coupled platelet P2Y6/P2Y12 and endothelial or lymphoid P2Y1/P2Y2 receptors, as well as via lymphoid “cytolytic” P2X7 and other P2X receptors with intrinsic pore-forming activities (6, 13).

Endothelial E-type NTP-diphosphohydrolases (E-NTP-Dases,1 otherwise known as ecto-ATPDase, CD39), in concert with ecto-5′-nucleotidase/CD73, represent the important effector system for maintenance of anti-thrombotic and anti-inflammatory state in the vasculature by rapid nucleotide inactivation via stepwise reactions ATP → ADP → AMP → adenosine (8, 12, 16). The generated adenosine in turn has a non-redundant countering role in the attenuation of inflammation and protection from excessive tissue damage in vivo via interaction with its own nucleoside-selective receptors (5, 17). Particularly, adenosine stimulates angiogenesis and acts as a potent cytotoxic agent (18), maintains vascular endothelial (19, 20) and intestinal epithelial (21) barrier functions, inhibits the release of cytokines from endothelial cells and the expression of E-selectin and other adhesion molecules (22), and suppress neutrophil function by interfering with their adherence to endothelial cells (23). Subsequent to signal transduction, adenosine needs to be inactivated either by rapid uptake into the cells (24) or via deamination by ecto-adenosine deaminase usually associated in larger complexes with CD26/dipeptidyl peptidase IV (25). Along with the purine-inactivating pathway, an alternative possibility of ATP resynthesis via sequential ecto-adenylate kinase- and ecto-NTPDase-mediated reactions has

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‡ To whom correspondence should be addressed: MediCity, Tykistökatu 6A, 20520 Turku, Finland. E-mail: genyeg@utu.fi.

1 The abbreviations used are: E-NTPDase, E-type NTP-diphosphohydrolase; CFSE, carboxyfluorescein diacetate, succinimidyl ester; HUVEC, human umbilical vein endothelial cells; PBL, peripheral blood lymphocytes; PI-PLC, phosphatidylinositol-specific phospholipase C; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; BCECF, bis-carboxyethyl carboxyfluorescein.
been recently described in our (10, 26) and other (3, 27–30) laboratories.

Given that endothelial and lymphoid cells are generally characterized by counteracting ATP-inactivating and ATP-regenerating/adenosine-removing phenotypes, respectively (10), these studies were designed to measure purine-converting pathways in the dynamics of leukocyte-endothelial interactions and, in this way, identify a link between the adhesion cascade and extracellular purine turnover.

EXPERIMENTAL PROCEDURES

Isolation and Treatment of Cells—HUVEC (passages 2–3) were grown to confluence either on 24-well tissue culture plates (Greiner) or Transwell polycarbonate membranes (0.4–5-μm pore sizes; Costar) as described previously (26). PBL were isolated from freshly drawn donor blood using Ficol centrifugations. The B cell lymphoma line Namalwa was from ATCC. In some experiments, the cells were preincubated with soluble apraxine from potato (grade III, 2 units/ml) or recombinant PI-PLC (0.5 units/ml) from Bacillus thuringiensis (both from Sigma), and then washed twice with RPMI 1640. The lymphocytes were also disrupted by sonicaton (Branson sonicator Labsonic U) and centrifuged for 10 min at 15,000 × g to obtain the conventional “crude membrane” (pellet) and cytosolic fractions. The cytosolic fractions were centrifuged through microcentrifuge tubes (mass cut-off 1 kDa; Pall Filtron) and additionally treated with 8% Norit A (ICN Biochemicals) for elimination of endogenous nucleotides.

Ectoenzymatic Interconversion of 3H-Nucleotides—Ecto-nucleotidas were assayed with [2-3H]AMP (specific activity 18.6 Ci/mmol; Amersham Biosciences) and [3H]ATP (19 Ci/mmol; Sigma) as reported previously (26). Ecto-5'-nucleotidase-depleted lymphoid cells were co-cultured at 37 °C under gentle orbital rotation in the following ways: (a) HUVEC monolayers were incubated 20 min with lymphocytes (or their subcellular fraction) and then either washed to remove non-adherent cells or directly challenged with [3H]-nucleotides; (b) Transwell-Cell cultures (3 μm) were placed onto a plate with cultured HUVEC. Namalwa cells (1 × 10^6) and THP-1 were added to the upper and lower chambers, respectively; (c) for 5'-nucleotidase kinetics, detached HUVEC (5 × 10^5 cells) were incubated in RPMI 1640 containing 5 mM β-glycerophosphate, AMP (20–900 μmol/liter) with tracer [3H]AMP and other nucleotides or lymphocytes. Adenine nucleotides/nucleosides were separated by thin layer chromatography and quantified by scintillation counting (31).

Quantification of Extracellular ATP—Cultured HUVEC and/or Namalwa cells (1.5 × 10^6 per well) were incubated in 24-well plates, and extracellular ATP was quantified by luciferin-luciferase assay as described previously (26).

Lymphocyte Binding to Endothelial Cells—Namalwa cells were incubated for 30 min at 37 °C in RPMI 1640 containing 15 μg/ml bis-carboxyethyl carbazoxol fluorescein, acetoxymethyl ester (BCECF) (Lamb-da Fluorescenttechology) and 5% fetal calf serum and rinsed to remove non-bound dye. BCECF-labeled lymphocytes were incubated with HUVEC monolayers for 60 min at 37 °C under orbital rotation in the final volume of 250 μl of RPMI 1640. After intensive washing, the adherent lymphocytes were detached by using 5 mM/liter EDTA, harvested into white non-phosphorescent microplate (Clinilate), and quantified with a TECAN Ultra fluoropolarimeter (excitation/emission wavelengths 485/545 nm).

Paracellular Permeability Assay—Using a modification of the method previously described (29), HUVEC monolayers grown on polycarbonate permeable inserts (0.4-μm pore size) were placed into 24-well plates containing 0.8 ml RPMI 1640. Namalwa cells (5 × 10^6 per well) were added to inserts and preincubated for 20 min in the final volume of 200 μl of RPMI 1640 without or with 50 μmol/liter exogenous adenosine. The assay was started by addition of 3.5 μmol/liter FITC-dextran (70 kDa; Sigma) in PBS (1×) to fluid within the insert, and the plates were incubated at 37 °C under orbital rotation (60 rpm). Aliquots of the assay medium from the lower chamber were transferred to the white 96-well microplate and assayed for FITC-dextran concentration (excitation, 485 nm; emission 545 nm; TECAN Ultra).

Transmigration Studies—For transmigration studies, Namalwa cells were labeled by incubation for 10 min at 37 °C with 10 μmol/liter CFSE (Molecular Probes) in 1 ml of PBS containing 1% bovine serum albumin. HUVEC monolayers grown on permeable inserts (5 μm pore size) were placed into 24-well white Visiplate (PerkinElmer Life Sciences) containing 0.9 ml of RPMI 1640 medium and 50 ng/ml B-lymphocyte chemotactrant (R&D Systems). The inserts were preincubated for 20 min with 100 μl of RPMI 1640 (alone or with 50 μmol/liter adenosine) prior to addition of lymphocytes. Meantime, CFSE-labeled Namalwa were also treated for 20 min in the absence or presence of adenosine (50 μmol/liter) followed by direct transfer of 100 μl of lymphocyte suspension (5 × 10^6 cells) to the insert with HUVEC monolayers. The plates were gently rotated for 3.5 h at 37 °C, and fluorescence in the lower chamber was directly measured by using TECAN-ULTRA fluoropolarimeter (excitation, 485 nm; emission, 545 nm). To correct for the nonspecific leakage of the fluorescent dye, CFSE-labeled lymphocytes were also incubated in parallel 0.4 μM Transwells, and this background fluorescence was taken into account during calculation of the number of transmigrated lymphocytes.

Dot-blot and Immunoblotting Analyses—HUVEC monolayers were incubated with HUVEC monolayers for 60 min at 37 °C before harvesting the cells, and extracellular purine turnover. The medium was collected, and soluble CD73 molecules were determined by dot-blot assay with anti-CD73 mAb 4G4 as described (32). HUVEC with adherent lymphocytes were pooled from four similar wells and subjected to 8% SDS-PAGE.

Because Namalwa-challenged HUVEC contained adherent lymphocytes, the amounts of the lysate loaded per lane were adjusted per number of endothelial cells in the wells rather than per total protein amount. Ecto-5'-nucleotidase was detected by using rabbit polyclonal antibody against human ecto-5'-nucleotidase (gift from Dr. Joseph Sypcha) and normal rabbit serum (1:100) as a negative control, as described previously (10).

Cell Surface Bioporation—The endothelial surface proteins were biotinylated either before or after the incubation of cultured HUVEC with Namalwa cells. The cells were incubated for 40 min at 37 °C in a final volume of 2 ml of phosphate-buffered saline containing 1 mg/ml sulfo-N-hydroxysuccinimido-biotin (Pierce), 0.1 mM/liter CaCl_2, and 1 mM/liter MgSO_4. Cells were solubilized in a lysis buffer containing 50 mM/liter β-mercaptoethanol, 1 mM/liter phenylmethylsulfonyl fluoride, and 1% aprotinin (all from Sigma), and the lysates were centrifuged for 40 min at 20,000 × g and 4 °C. The biotin-labeled surface proteins were precipitated with immobilized monomeric avidin beads (Roche Molecular Biochemicals) and then eluted from streptavidin pellets with the SDS sample buffer and subjected to 8% SDS-PAGE. After electrophoresis, the membrane was precut to separate each sample at the top to avoid overlap of bands, and each sample was transferred onto nitrocellulose and analyzed with a fluorescence microscope (Olympus BX60).

Fluorescence Microscopy—Cultured HUVEC were incubated for 30 min at 37 °C with or without Namalwa (1.5 × 10^6 cells), washed, and stained with the following mABs: 1E9 (gift from Dr. Linda Thompson) and 4G4, both recognizing different epitopes of CD73 but partially inhibiting its catalytic activity; 2C8 against endothelial CD31; and 3G6 against chicken T-cells (32). For intracellular staining, HUVEC were first fixed with 4% paraformaldehyde and permeabilized by using 0.2% Triton X-100. The second-stage antibody used was a FITC-conjugated anti-mouse Ig (DAKO A5), and the slides were mounted with Fluoromount and analyzed with a fluorescence microscope (Olympus BX60).

Data Analyses—Statistical comparisons were made using Student’s t test, and p values < 0.05 were taken as significant. Ecto-5'-nucleotidase kinetic parameters (K_m and V_max) were calculated by using the Michaelis-Menten equation (GraphPad Prism®; version 3.09; San Diego, CA).

RESULTS

Lymphocytes Inhibit Endothelial Ecto-5'-nucleotidase but Not E-NTPDase—In the initial assays, ecto-5'-nucleotidase activity was assayed in cultured HUVEC and PBL suspension and defined as the rate of [3H]AMP conversion into [3H]nucleotides. Both cell types displayed significant AMP-hydrolyzing activity equal to 264 ± 31 (n = 22) and 8.3 ± 1.1 (n = 14) nmol/10^6 cells/hour, respectively. Surprisingly, co-incubation of HUVEC with PBL was accompanied by decrease of the joint 5'-nucleotidase activity by 18.2 ± 1.2% (n = 12; p < 0.01) as compared with the theoretical sum value of both cell types taken alone (Fig. 1A).

Because understanding of the underlying inhibitory mechanisms is hampered by co-expression of ecto-5'-nucleotidase, we pretreated either endothelial or lymphoid cells with PI-PLC (0.5 units/ml), thereby causing shedding of at least 75–80% of the glycosyl-phosphatidylinositol-anchored 5'-nucleotidase molecules (10). Addition of partially 5'-nucleotidase-depleted PBL to the HUVEC still decreased the joint AMP hydrolysis,
whereas no significant inhibition was observed after PBL addition to the PLC-treated HUVEC (data not shown). Moreover, ecto-5’-nucleotidase/CD73-negative Namalwa B-cells (Fig. 1A) and Jurkat T-cells (data not shown) inhibited endothelial ecto-5’-nucleotidase even more efficiently as compared with PBL. Together, these data suggest that the inhibition is primarily because of leukocyte-mediated inhibition of endothelial ecto-5’-nucleotidase and that it is not restricted to a certain lymphocyte subpopulation. Use of other nucleotide substrates, [3H]ATP (Fig. 1B) and [3H]ADP (data not shown), did not reveal significant changes in the joint E-NTPDase activities during leukocyte-endothelial interaction.

Role of Lymphocyte-derived Nucleotides in Endothelial Ecto-5’-nucleotidase Inhibition—For further experiments we took advantage of the ability of Namalwa cells to inhibit endothelial 5’-nucleotidase in a concentration-dependent fashion (Fig. 2A).

After lymphocyte disruption, only cytosolic extracts, but not membrane pellet, are able to inhibit endothelial enzyme, although not as efficiently as intact cells. The cytosolic fraction retained its inhibitory potency after passage through low-molecular mass cut-off filters (Fig. 2A), whereas the removal of endogenous nucleotides by Norit A prevented its inhibition of endothelial 5’-nucleotidase (97.2 ± 1.9% of the control activity; n = 2). Direct bioluminescent assay confirmed the presence of relatively high ATP concentrations in Namalwa lysates (933 ± 134 pmol/10^6 cells, n = 11).

Although these data indicate that lymphocyte-derived nucleotides may in principle act as inhibitors of endothelial 5’-nucleotidase, a distinction needs to be drawn between the inhibitory effects of lymphoid cells and their lysates. In the case of intact cells, an alternative mechanism for the nucleotide appearance could be the nonlytic release of endogenous ATP. Both HUVEC and Namalwa maintained ATP at a certain steady-state level as detected by luciferin-luciferase assay (Fig. 2B). However, because inhibition of endothelial 5’-nucleotidase is only detectable at threshold ATP/ADP concentrations of ~10^{-5} mol/liter (26), it seems unlikely that nanomolar levels of the released ATP are sufficient to block [3H]AMP hydrolysis in our studies. Removal of extracellular ATP by Namalwa treatment with apyrase did not prevent their inhibitory action on endothelial 5’-nucleotidase, and, furthermore, only slight enzyme inhibition was observed when Transwell chambers with lymphocyte suspension were placed on the top of the wells with cultured HUVEC (Fig. 2C). These data suggest the involvement of other cellular mechanisms in the enzyme inhibition by intact lymphocytes.

Endothelial Ecto-5’-nucleotidase Is Primarily Inhibited via Lymphocyte Adhesion—Incubation of HUVEC with Namalwa was accompanied by stable lymphocyte adhesion on the endothelial surface with prominent cluster formation (Fig. 3A). For quantitative analysis, Namalwa cells were labeled with fluorescent dye BCECF. Non-linear regression analysis revealed that lymphocyte binding to the HUVEC fits to a sigmoidal curve (Fig. 3B) with maximum binding capacity of 243 ± 29 ×
10^6 cells per well (n = 5). This is equivalent to about four lymphocytes per one endothelial cell. Importantly, the number of adherent lymphocytes shows a highly significant correlation with their ability to inhibit endothelial 5'-nucleotidase (Fig. 3C), thus allowing us to consider the enzyme inhibition as a direct consequence of lymphocyte binding.

**Kinetic Evidence That Lymphocytes and Their Lysates Inhibit Endothelial 5'-nucleotidase via Different Mechanisms**—Kinetic analysis of [3H]AMP hydrolysis by HUVEC was then performed in the presence of fixed amounts of lymphocytes and other nucleotides as potential 5'-nucleotidase inhibitors. Inhibitor concentrations were chosen to approximate IC_{50} values found in preliminary competitive experiments with Namalwa cells (see Fig. 2A) and the major enzyme-inhibiting nucleotides AMPCP (α,β-methylene ADP), ADP, and ATP (26).

Statistical analysis revealed a decrease of the maximal velocity (V_max) of endothelial 5'-nucleotidase in the presence of lymphocytes (Fig. 4B) without any changes in the apparent K_m values (Fig. 4A), thus indicating that these inhibitory effects are non-competitive in nature. Contrary to native lymphocytes, their cytosolic fractions primarily affected the enzyme affinity (manifested in the increased K_m value), and this competitive mechanism was similar to that observed with exogenous nucleotides (Fig. 4). These data confirm the existence of two distinct and principally independent inhibitory mechanisms including 1) non-competitive inactivation of endothelial 5'-nucleotidase by adherent lymphocytes and 2) leakage of endogenous NTP/NDP under cell damage and their competition with AMP for the enzyme catalytic site.

**Lymphocytes Switch Endothelial Purine Metabolism from Adenosine Formation to ATP Re-synthesis via Phosphotransfer Reactions**—To evaluate the whole pattern of purine metabolism, HUVEC monolayers and/or Namalwa cells were incubated with 10 μmol/liter [3H]AMP; Fig. 5A depicts a representative thin layer chromatography autoradiography of its major interconversion pathways. HUVEC 5'-nucleotidase efficiently converted AMP into nucleosides with adenosine being the major metabolite (lane 2). Co-incubation of Namalwa with HUVEC prevented adenosine appearance, first via inhibition of 5'-nucleotidase-mediated AMP hydrolysis and second, by rapid adenosine conversion via inosine to hypoxanthine (lane 4). Inhibitor of adenosine deaminase erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) blocked further conversion of adenosine in the experiments with HUVEC, either alone (lane 3) or in combination with lymphocytes (lane 5). These findings are consistent with our recent studies showing that a significant portion of [3H]adenosine is directly inactivated by the lymphoid surface (10). Notably, the amount of radioactivity retained by lymphocytes during their incubation with HUVEC and [3H]AMP did not exceed 0.5–0.7% of total radioactivity added. This value was not affected by inhibitors of the nucleoside transport NBTI and dipyridamole (data not shown), thus allowing us to exclude possible uptake of nucleotide-derived adenosine into the cells.

Although Namalwa were unable to degrade [3H]AMP, these cells were capable of converting [3H]AMP into [3H]ADP (Fig. 5A, lane 6). Further time-course analysis revealed that this...
[14]AMP phosphorylation was only partially inhibited after lymphocyte pretreatment with soluble apyrase as well as during co-incubation of Namalwa cells with HUVEC (Fig. 5B). Because this backward AMP phosphorylation is mediated via ecto-adenylate kinase reaction and requires co-incubation of Namalwa cells with HUVEC (Fig. 5B), Namalwa alone (filled circles), HUVEC alone (open circles), HUVEC plus Namalwa cells (open squares), Namalwa were also pretreated with 2 units/ml soluble apyrase prior to [13H]AMP addition (open triangles). The graphs show mean data of two independent experiments that differed by less than 10%.

Inactivation of Endothelial Ecto-5'-Nucleotidase/CD73 by Lymphocytes Does Not Involve Enzyme Internalization, Sequestration, or Sheddning—Because ecto-5'-nucleotidase on various cell types is attributed to the glycosyl-phosphatidylinositol-anchored surface antigen CD73 sharing both enzymatic and antigenic properties (32, 33), we performed immunofluorescent staining to follow the fate of the endothelial ecto-5'-nucleotidase/CD73 molecule; these data are summarized in Fig. 6. Cultured HUVEC show intense staining with anti-CD73 antibody 4G4 in a pattern indicative of membrane localization of this antigen (panel A), and it became relatively faint after lymphocyte binding to HUVEC (panel B). Use of another anti-CD73 mAb, 1E9, gave similar staining results (data not shown). The differences in the CD73-specific staining between control (panel C) and lymphocyte-treated (panel D) HUVEC still remained evident after permeabilization of the cells, thus excluding the possibility of endothelial ecto-5'-nucleotidase/CD73 internalization during lymphocyte binding. Such lymphocyte-mediated inhibition is specific for CD73, because the expression of another endothelial molecule CD31 remained unchanged when HUVEC were incubated in the absence (panel E) or presence (panel F) of Namalwa cells.

Lymphocyte binding did not stimulate any shedding of 5'-nucleotidase/CD73 from the HUVEC surface, whereas use of PI-PLC treatment as a positive control caused massive release of soluble CD73 molecules into the bathing medium (Fig. 7A). Next, we wanted to examine whether the leukocyte-mediated inhibition of endothelial ecto-5'-nucleotidase/CD73 is accompanied by concurrent loss of surface-associated enzyme itself. Because the available monoclonal anti-CD73 antibodies are not suitable for immunoblotting analysis, we used a polyclonal antibody against a peptide corresponding to residues ETP-FLSNPGTNLVFGD of the human ecto-5'-nucleotidase (10), and a single protein band of ~70 kDa was clearly detected in HUVEC lysates (Fig. 7B). The total pool of ecto-5'-nucleotidase molecules remained unchanged after HUVEC incubation with Namalwa (Fig. 7B). Similar results were observed when surface CD73 was biotinylated prior to addition of lymphocytes (Fig. 7C). Importantly, co-incubation of HUVEC and Namalwa with subsequent biotinylation of cell surface proteins revealed significant decrease in the amount of the biotinylated ecto-5'-nucleotidase molecules (Fig. 7C). These data suggest that lymphocytes mask endothelial ecto-5'-nucleotidase without any covalent modification of this ectoenzyme or its sequestration within plasma membrane subdomains.

Adenosine Is Important for Regulation of Vascular Barrier Function and Transendothelial Leukocyte Migration—Permeability assays and transmigration studies were then performed by co-incubation of Namalwa suspension with HUVEC monolayers grown on permeable inserts. The cells were pretreated with or without 50 μmol/liter adenosine, thus allowing us to compensate the loss of endogenous adenosine that occurs during leukocyte-endothelial interactions and, in this way, to eval-

![Fig. 5. Pattern of [14]H]AMP metabolism by endothelial and lymphoid cells. A, HUVEC monolayers and/or Namalwa suspension (1 × 10⁶ cells) were incubated for 30 min with 10 μmol/liter [14]H]AMP in the absence or presence of 10 μmol/liter erythro-9(2-hydroxy-3-nonyl)adenine hydrochloride, as indicated. Note, the amount of radioactivity in this particular illustrative experiment was 8–10 times higher (~3 × 10⁵ dpm/lane) than that used in the quantitative assays. B, the cells were also incubated with 50 μmol/liter [14]H]AMP. Aliquots of the medium were assayed for the relative amounts of phosphorylated [14]H-derivatives. The cells were co-cultured in the following combinations: HUVEC alone (open circles), Namalwa alone (filled circles), HUVEC plus Namalwa cells (open squares). Namalwa were also pretreated with 2 units/ml soluble apyrase prior to [14]H]AMP addition (open triangles). The graphs show mean data of two independent experiments that differed by less than 10%.

![Fig. 6. Effect of lymphocytes on HUVEC CD73 and CD31 staining.](Image) Cultured HUVEC were incubated without (A, C, E) or with (B, D, F) Namalwa cells and washed to remove non-adherent lymphocytes. HUVEC were stained with an anti-CD73 mAb 4G4 (A, B) or anti-CD31 mAb 2C8 (E, F). For intracellular CD73 staining, HUVEC were fixed and permeabilized with saponin prior to staining with 4G4 (C, D). The isotype control mAb 3G6 shows only background staining (panel F, inset). Bars, 25 μm.
valuate the physiological consequences of the leukocyte-mediated suppression of endothelial metabolism. The paracellular permeability assay was first performed by using 0.4-μm Transwells and FITC-labeled dextran (70 kDa). Exogenous adenosine significantly decreased the clearance of FITC-dextran across inserts with endothelial monolayers co-incubated without or with lymphocyte suspension (Fig. 8A). Further transmigration studies revealed that the number of CFSE-labeled Namalwa cells that had migrated across the HUVEC-containing Transwells containing exogenous adenosine was lower when compared with untreated cells (Fig. 8B). Noteworthy, both clearance of FITC-dextran (Fig. 8A) and lymphocyte transmigration (Fig. 8B) across the empty inserts were higher compared with the HUVEC monolayers, thus confirming that endothelial cells in fact serve as a barrier preventing paracellular flux of macromolecules and restraining cell migration.

**DISCUSSION**

By investigating the combined features of lymphocyte-endothelial interactions and extracellular purine metabolism, we have identified a link between these different but apparently interrelated processes and have additionally shown that endothelial ecto-5'-nucleotidase/CD73 is a potential key target for circulating lymphocytes. Namalwa B-cells caused progressive inhibition of endothelial ecto-5'-nucleotidase activity with a concomitant decrease in the CD73-specific staining of the HUVEC surface. The extent of ecto-5'-nucleotidase inhibition showed a close correlation with the number of adherent lymphocytes, thus allowing us to consider the enzyme inhibition as a direct consequence of lymphocyte-endothelial adhesion. This inhibition was specific for ecto-5'-nucleotidase/CD73 and did

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**FIG. 7. Immunoblotting analysis of HUVEC ecto-5'-nucleotidase/CD73.** A, HUVEC were incubated with Namalwa cells (1 × 10⁶ per well), and the medium was assayed for soluble CD73 by a dot-blot with an anti-CD73 mAb 4G4 and the isotype control mAb 3G6. As an assay control, HUVEC were pretreated with PI-PLC (0.5 units/ml), causing massive release of soluble CD73 to the medium. B, HUVEC monolayers were preincubated with or without Namalwa (1 × 10⁶ cells) followed by immunoblotting with a polyclonal anti-ecto-5'-nucleotidase antibody (α-5-NT) or normal rabbit serum as a negative control. C, to cause the biotinylation (Biot) of cell-surface proteins, HUVEC were subjected to the following treatments: biotin alone, biotin followed by incubation with Namalwa, or preincubation with Namalwa followed by biotin treatment. The biotinylated ecto-5'-nucleotidase molecules were then immunoprecipitated with streptavidin followed by Western blotting with α-5-NT polyclonal antibodies. The arrows indicate the position of the molecular mass markers (kDa). All data are representative of three independent experiments with similar results.

**FIG. 8. Effect of adenosine on endothelial permeability and lymphocyte transmigration.** A, HUVEC monolayers were grown on 0.4-μm Transwells and preincubated for 20 min with Namalwa suspension and/or adenosine, as indicated. Paracellular permeability assay was initiated by addition of 3.5 μmol/liter FITC-dextran 70 kDa to the inserts followed by measurement of its flux to the lower chamber (mean ± S.E., n = 4–5). B, Transwells (5-μm pore size) with or without cultured HUVEC were incubated for 3.5 h with CFSE-labeled Namalwa cells and exogenous adenosine. The bars show the number of lymphocytes migrated to the lower chamber (mean ± S.E., n = 3). The number of lymphocytes in all studies was equal to 5 × 10⁵ cells, and adenosine concentration was 50 μmol/liter. *, p < 0.05 as compared with control.
not affect the activity of another endothelial ectoenzyme, E-NTPDase, or the expression of the HUVEC CD31 molecule. Noteworthy, freshly isolated PBL were also able to inhibit endothelial ecto-5'-nucleotidase, although not as efficiently as leukemic cells. These relatively slight inhibitory effects may be because of co-expression of ecto-5'-nucleotidase/CD73 and heterogeneity of PBL with significant variations in the CD73 expression among lymphocyte subsets (33).

Taking into account the existence of highly efficient shuttle mechanisms between extra- and intracellular 5'-nucleotidase pools in some cell types (32, 34), one might suggest a down-regulation of the endothelial enzyme under lymphocyte adhesion via rapid internalization and/or prevention of its recycling on the cell surface. However, this seems unlikely because at least 85–90% of ecto-5'-nucleotidase/CD73 molecules are located on the HUVEC surface (10). Moreover, HUVEC permeabilization did not provide evidence for endothelial enzyme accumulation in intracellular stores upon lymphocyte adhesion. Alternatively, the enzyme may be shed from the cell surface, as was demonstrated earlier with glycosyl-phosphatidylinositol-anchored ecto-5'-nucleotidase/CD73 and other purine-converted ectoenzymes during mAb triggering (32), shear dylinositol-anchored 5'-nucleotidase/CD73 and other pu-rinase/CD73 molecule and inhibition of its catalytic activity by adherent lymphoid cells directly on the cell surface, presumably via a temporal and non-competitive mechanism.

We also revealed yet another mechanism of competitive ecto-5'-nucleotidase inhibition by endogenous nucleotides, but it occurs only after the disruption of lymphocytes. Although these data fit well with the known feed-forward inhibition of endothelial 5'-nucleotidase by micromolar concentrations of precursor ATP/ADP (8, 26), this inhibitory mechanism is presumably restricted to sites of traumatic tissue injury with massive nu-cleotide release from the damaged cells and/or dense granules of activated platelets.

In parallel with inhibition of endothelial ecto-5'-nucleotidase/CD73, lymphocytes efficiently deaminated nucleotide-de-rived adenosine, thereby providing a supplementary scaveng-ing mechanism that prevented adenosine appearance during lymphocyte-endothelial adhesion. An important point of considera-tion is whether this leukocyte-mediated elimination of adenosine via the dual mechanism of inhibition of endothelial ecto-5'-nucleotidase and catalytic deamination of the remaining nucleoside is relevant to basal and/or acute inflammatory responses in vitro. Extracellular adenosine has been shown to enhance vascular barrier function by activating endothelial A2B receptors (19, 20) and to interfere with leukocyte-endothelial adhesion via occupancy of A1 receptors on stimulated neutrophils (17, 23). Because the released adenosine nucleotides provide the major source of extracellular adenosine, directional regulation of ecto-5'-nucleotidase-mediated AMP hydrolysis significantly affects permeability changes in the vascular en-dothelium (20) and intestinal epithelial cells (21) and inhibits neutrophil function at inflamed sites in the murine air porch model of inflammation (36). Our permeability and transmigra-tion studies confirm the important role of adenosine in the maintenance of vascular barrier function and further demon-strate for the first time that selective inhibition of endothelial ecto-5'-nucleotidase activity occurs in the dynamics of leuko-cyte-endothelial adhesion, and it may serve as a prerequisite for facilitation of subsequent leukocyte transmigration into the tissue.

Another relevant point is that, because ecto-5'-nucleotidase serves as a “master switch” between two counteracting, ATP-consuming and ATP-generating extracellular pathways (26), inhibition of this endothelial ectoenzyme by adherent lymphocytes directionally shifts the joint purine metabolism toward continuous ATP regeneration via phosphotransfer reactions. By using luciferase-based luminometric assay, we showed that lymphoid cells steadily maintain extracellular ATP at a certain nanomolar range that basically represents a net balance between the constitutive release of endogenous ATP and/or its ectoenzymatic interconversion (3). Noteworthy, incubation of Namalwa cells with [3H]AMP caused its significant transphosphorylation via an ecto-adenylate kinase reaction that occurs even in the absence of exogenous γ-phosphate-donating ATP and remains resistant to treatment with apyrase (Ref. 10; see also Fig. 5).

Recent luminometric assays with firefly luciferase stably adsorbed onto the platelet surface (37); confocal microscopy studies with pancreatic acini (4) also showed transient formation of relatively high (micromolar) concentrations of apyrase-resistant percellular ATP that differ from the “bulk” extracellular ATP pool. The ability of lymphocytes to retain the elevated percellular ATP level may provide an efficient para-crine mechanism for leukocyte-governed triggering of purinergic signaling in the targeted endothelial cell.

In conclusion, the results presented here demonstrate that the pattern of purine metabolism is selectively disturbed during leukocyte adhesion, thereby affecting the functional state of the targeted endothelium and leading to a preponderance of pro-inflammatory mechanisms. These findings provide a novel insight into the leukocyte adhesion-mediated changes of vascular endothelium such as disorganization of inter-endothelial junctions (38), impairment of barrier function and promotion of transendothelial leukocyte migration (this study), and transfer of Ca2+ fluxes to the counter endothelial cells (39).

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