

Protein, not adenosine or adenine nucleotides, mediates platelet decrease in endothelial permeability

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Patil, Sandeep, John E. Kaplan, and Fred L. Minnear. Protein, not adenosine or adenine nucleotides, mediates platelet decrease in endothelial permeability. *Am. J. Physiol.* 273 (*Heart Circ. Physiol.* 42): H2304–H2311, 1997.—Platelets and platelet-conditioned medium (PCM) decrease endothelial protein permeability in vitro. Adenosine and a >100-kDa protein have previously been implicated as the soluble factors released from platelets that decrease endothelial permeability. The objective of this study was to further investigate the role of adenosine in this platelet response. Measurements of adenosine and its precursor adenine nucleotides by high-performance liquid chromatography were correlated with the assessment of permeability by ¹²⁵I-labeled albumin clearance and electrical resistance across endothelial cell monolayers derived from the bovine pulmonary artery. PCM contained micromolar concentrations of AMP, ADP, and ATP, but adenosine was below detectable levels ($\leq 0.1 \mu\text{M}$). Adenosine deaminase, an enzyme that converts adenosine to inactive inosine, or an adenosine-receptor antagonist did not block the platelet- or PCM-mediated decrease in endothelial permeability. A <3-kDa fraction of PCM that contained micromolar concentrations of AMP and ADP did not affect endothelial permeability, whereas a >3-kDa fraction that contained much reduced levels of AMP and ADP significantly decreased permeability. This activity of PCM was sensitive to insoluble trypsin. This study rules out adenosine and adenine nucleotides as primary factors in the platelet-induced decrease in endothelial permeability and suggests that the active factor is a protein.

high-performance liquid chromatography; albumin clearance; electrical resistance; adenosine deaminase; adenosine-receptor antagonist; trypsin

BLOOD PLATELETS PLAY a central role in hemostasis. Platelets also appear to contribute to the maintenance of the vascular endothelium as a semipermeable membrane to the passage of water and protein. Experimental and clinical evidence show that thrombocytopenia (platelet count 50,000/ μl or less) results in disruption of the microvascular endothelium in such a way as to cause nontraumatic petechial and purpuric hemorrhages in the skin and mucous membranes (10, 15) and to increase protein permeability in the lung (17), ear (2), thyroid (12), and coronary microvasculature (18). A transfusion of platelets reverses these abnormalities (2, 12, 15, 17, 18). Three major theories have been put forth to explain the platelet effect: 1) platelets physically block pores or gaps in the vascular lining (9), 2) platelets or platelet components promote the growth of endothelial cells (8), and 3) platelets release soluble factors that modulate the permeability of the vascular lining cells (13, 22, 24). Although it is recognized that these theories are not mutually exclusive, the present paper focuses on evaluating and defining the last

theorized mechanism. Platelet-conditioned medium (PCM), which contains releasate from platelet granules, can replicate the permeability-decreasing effect of whole platelets (22, 24). Three potential factors in PCM have been proposed to decrease endothelial permeability, i.e., a low-molecular-weight factor, possibly adenosine (22); a <10-kDa molecule (29); and a >100-kDa protein (13).

There is evidence in the literature both for and against the role of adenosine as the primary factor responsible for the permeability-decreasing activity of platelets on the vascular endothelium (13, 22). Adenosine, a potent vasodilator, is known to decrease endothelial protein permeability in vitro and in vivo (1, 14, 22). This response is mediated primarily by A₂-purinergic receptors on endothelial cells that, when activated, increase the intracellular level of adenosine 3',5'-cyclic monophosphate (1, 14). Adenosine deaminase, which metabolizes adenosine to inactive inosine, has been used in two studies to determine the importance of adenosine in the permeability-decreasing activity of platelets. Paty et al. (22) blocked this activity of platelets after treatment with 100 U/ml of adenosine deaminase, whereas Haselton and Alexander (13) showed that PCM retained its activity after treatment with 2.5 U/ml of the same enzyme.

The objective of the present study was to determine further whether adenosine is the primary factor released from platelets that decreases the vascular endothelial permeability to protein. The ability of PCM to decrease endothelial permeability, as assessed by the clearance of ¹²⁵I-labeled albumin and electrical resistance across cell monolayers, was tested after incubation with adenosine deaminase and an adenosine-receptor antagonist, after separation of PCM by dialysis or microconcentration into <3- and >3-kDa fractions, and after incubation with insoluble trypsin. Analytic reverse-phase high-performance liquid chromatography (HPLC) was used to determine the concentration of adenosine in PCM, the dose of adenosine deaminase sufficient to metabolize 10 μM adenosine added exogenously to PCM, the retention of adenine nucleotides in the <3-kDa fraction, and the lack of adenine nucleotides in the >3-kDa fraction.

METHODS

Platelet isolation. Platelets were isolated by a modification of the methods of Corash et al. (7) and Moon et al. (21) from either whole human blood or fresh, nonirradiated platelet packs purchased from the local blood bank. This method produced platelet concentrates containing $1\text{--}4 \times 10^9$ platelets/ml. The effective concentration of platelets that decreased albumin clearance by ~40–60% of the control level and

increased electrical resistance by 30% was $2-10 \times 10^7$ platelets/ml.

Preparation and fractionation of PCM. Isolated platelets were brought to a concentration of 1×10^9 platelets/ml in modified (Ca^{2+} - Mg^{2+} -free) Tyrode buffer (0.137 M NaCl, 0.003 M KCl, 0.012 M NaHCO_3 , 0.006 M glucose, and 0.004 M NaH_2PO_4 , pH 7.4), incubated in a plastic, round-bottom tube for 2 h at room temperature, and centrifuged at 600 *g* for 20 min. The supernatant was designated PCM. PCM was separated into fractions based on molecular mass by microconcentration or dialysis. A microconcentrator (Amicon, Beverly, CA) with a 3-kDa molecular-mass cutoff was used to fractionate the PCM. The PCM (2–4 ml) was also dialyzed 1:100 against modified Tyrode buffer at 4°C for 3 days with Spectra/Por dialysis membranes (Spectrum Medical Industries, Los Angeles, CA) with molecular-mass cutoffs of 3.5, 12–14, 50, and 100 kDa. After separation by microconcentration or dialysis, the PCM was aliquoted and stored at –80°C before use.

HPLC. The presence of adenosine and the adenine nucleotides ATP, ADP, and AMP in PCM was determined with isocratic reverse-phase HPLC with a Waters Resolve C₁₈ (5- μm , spherical) column (Millipore, Milford, MA) (28). Individual 10^{-3} M stock solutions of adenosine, ATP, ADP, AMP, and inosine (Calbiochem, La Jolla, CA) were prepared and volumetrically diluted in Milli-Q water. The moving-phase buffer was degassed with 0.22 M phosphate buffer (pH 6.8). The column was monitored by absorption at 259 nm, and the flow rate was 1 ml/min. Individual standard curves for adenosine, ATP, ADP, AMP, and inosine were constructed with the use of known concentrations of standards injected versus the observed chromatographic peak areas. Retention time and peak areas were used to identify and quantify, respectively, the generated ATP, ADP, AMP, adenosine, and inosine.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Analytic sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done with 4–15% gradient polyacrylamide minislab gels (Bio-Rad Mini-Protein II system, Hercules, CA) according to the method of Laemmli (16). Protein samples were diluted 1:2 in reducing and nonreducing sample buffers and added in a final volume of 20–40 μl /lane. Molecular-mass determinations were made with the following markers (from GIBCO BRL): myosin (200 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β -lactoglobulin (18.4 kDa), and lysozyme (14.3 kDa).

Experimental treatments of PCM. PCM was incubated with adenosine deaminase, BW-A1433U83 (an adenosine-receptor antagonist), and insoluble trypsin and separated into <3- and >3-kDa fractions as described in *Preparation and fractionation of PCM*.

The ability of 2.5 U/ml of adenosine deaminase to metabolize within 30 min a known concentration (10 μM) of adenosine to inactive inosine was verified by HPLC. The biological activity of this adenosine deaminase (2.5 or 10 U/ml)-treated PCM was assessed in endothelial cell monolayers within a 30-min experimental period by the clearance of ¹²⁵I-labeled albumin and electrical resistance. The adenosine-receptor antagonist BW-A1433U83 [dissociation constant (K_D) ~0.1 μM ; Burroughs Wellcome, Research Triangle Park, NC] (4) was added to PCM at a 10-fold molar excess in the presence of 0.1 and 1 μM adenosine or PCM to determine whether antagonism of the adenosine receptor prevented the permeability-decreasing activity of PCM. BW-A1433U83 is a *p*-phenylcarboxyl-substituted derivative of 1,3-dipropyl-8-phenylxanthine. It is 100 times more potent as a blocker of adenosine receptors than as an inhibitor of phosphodiesterase

activity (4). Insoluble trypsin was added to the PCM to determine whether a protein was the permeability-decreasing factor in the PCM. Trypsin cross-linked to beaded agarose (20 U/ml) was washed with Ca^{2+} - Mg^{2+} -free Tyrode buffer and then incubated with PCM for 10 min. Trypsin was removed by centrifugation, and the supernatant was designated as trypsin-treated PCM. As a sham control, trypsin was added to Tyrode buffer and removed by centrifugation. Trypsin was also added to endothelial cells and not removed by centrifugation to demonstrate its effect on endothelial permeability.

Cell culture. The bovine pulmonary artery endothelial cell line CCL-209 was obtained at the 16th passage from the American Type Culture Collection (Rockville, MD). These cells were grown in a complete culture medium consisting of Dulbecco's modified Eagle's medium (DMEM), 20% fetal bovine serum, 10 mM nonessential amino acids (all from GIBCO, Grand Island, NY), and 50 $\mu\text{g}/\text{ml}$ of gentamicin sulfate (Bioproducts, Walkersville, MD). Endothelial cells were seeded at the 19th–24th passages onto sterilized Transwell filters (75,000 cells onto 0.33-cm² and 0.4- μm pore filters; Costar, Cambridge, MA) for protein permeability experiments, onto 1% gelatin-coated gold electrodes (40,000 cells onto 0.5-cm² wells; Applied Biophysics, Troy, NY) for electrical resistance measurements, or onto 35-mm petri dishes (300,000 cells) for HPLC experiments and were then grown to confluence (3–4 days). Confluence of cell monolayers was tested routinely by fluorescent staining with calcein acetoxymethyl ester (Molecular Probes, Eugene, OR), a cytoplasmic vital dye that diffuses into the cytoplasm and is cleaved by intracellular esterases to release the polar, fluorescent compound calcein.

Measurement of ¹²⁵I-labeled albumin clearance. The experimental apparatus used allowed for the measurement of the transendothelial clearance of ¹²⁵I-labeled albumin in the absence of hydrostatic and oncotic pressure gradients and resembled a modified chemotaxis chamber (6, 19, 20). A luminal chamber (1 ml) contained the confluent, endothelial cell monolayer seeded on a polycarbonate membrane. This luminal chamber was kept afloat by a styrofoam collar in a stirred 25-ml abluminal chamber. A concentration gradient of ¹²⁵I-labeled albumin was established across the endothelial cell monolayer by placing 160 μl of DMEM containing tracer albumin, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, and 0.5% albumin into the luminal compartment. The experimental protocol consisted of adding 40 μl of either Tyrode buffer (medium) or the desired mediator(s) and then taking 400- μl samples from the lower compartment every 5 min for the 30-min duration of the experiment. Three twenty-five-microliter samples were obtained from the ¹²⁵I-labeled albumin stock at the beginning of each experiment to determine the initial radioactivity. Radioactivity of the samples was measured in a gamma counter (LKB/Wallace, Gaithersburg, MD). Albumin clearance was expressed as the volume of the luminal chamber that was cleared of albumin tracer into the abluminal chamber. The clearance of albumin over time was determined as described by Cooper et al. (6) with a weighted least squares nonlinear regression (BMDP Statistical Software, Berkeley, CA). The baseline clearance of albumin across endothelial cell monolayers ranged between 0.01 and 0.04 $\mu\text{l}/\text{min}$.

Bovine serum albumin was radioiodinated with the chloramine-T procedure as described by Bocci (3). After iodination, the ¹²⁵I-labeled albumin was separated from free ¹²⁵I by dialysis against normal saline (0.9%). Free ¹²⁵I was <0.1% in the luminal compartment at the beginning of the experiment as determined by comparing isotope stock with filtrate (CF 30; 30,000-molecular mass cutoff; Amicon).

Measurement of the resistive portion of electrical impedance. Measurement of the electrical impedance of endothelial cells grown as a monolayer is based on a technique developed to study the dynamic behavior of cells in culture. This novel methodology is known as electric cell-substrate impedance sensor (ECIS) (11, 26). Endothelial cells are cultured on small gold electrodes (10^{-3} cm²), and culture medium is used as the electrolyte. The small gold electrode, covered by confluent endothelial cells, and a larger gold counterelectrode are connected to a phase-sensitive, lock-in amplifier. A 1-V, 4,000-Hz alternating current signal is supplied through a 1-M Ω resistor to approximate a constant-current source of 1 μ A. Treating the cell-electrode system as a simple series resistance-capacitance circuit, the measured changes in electrical impedance can be partitioned into a measured in-phase voltage proportional to the resistance and an out-of-phase voltage proportional to capacitive reactance. Voltage and phase data are stored and processed with a personal computer. The same computer controls the output of the amplifier and switches the measurement to different electrodes in five different wells during the course of an experiment. Endothelial cells were grown in each of five wells containing the small gold electrode. ECIS wells were placed in the ECIS incubator for 30 min to equilibrate to 37°C and 5% CO₂ and then incubated with the desired mediators. The measurement of electrical impedance was obtained every minute for 30 min after treatment of the endothelial cells and reported, in the present study, as the resistive portion of electrical impedance.

Statistics. Each protein permeability study consisted of at least three experiments done on different days with five to eight cell monolayers per group per experiment. Each electrical resistance study consisted of at least five different experiments with one cell monolayer per group per experiment. The data were analyzed by a two-way analysis of variance (27). Differences between treatments or groups from the control group were analyzed with the least significant difference test. With those data that were normalized, the Shapiro-Wilk's test was used to confirm standard normal distribution of each of the dependent variables. When appropriate, log transformation was conducted on normalized data before analysis with analysis of variance. Statistical significance was set at $P < 0.05$.

RESULTS

HPLC was used initially to quantitate the amount of adenosine and adenine nucleotides present in PCM. The PCM profile on the chromatogram showed micromolar concentrations of the adenine nucleotides AMP (1.72 μ M), ADP (5.12 μ M), and ATP (0.56 μ M), but no adenosine was detected (Fig. 1A). It is possible that the PCM itself has some adenosine deaminase that would metabolize any adenosine into inactive inosine. To test this possibility, 10 μ M exogenous adenosine was added to the PCM, but it remained unmetabolized for 30 min (data not shown). These experiments demonstrate that adenosine was not present at detectable levels in PCM (≥ 0.1 μ M).

Because it is possible that an amount of adenosine below the sensitivity of our measurements (< 0.1 μ M) could be present in PCM or be generated when PCM was incubated with cell monolayers for 30 min, adenosine deaminase was added to metabolize adenosine to inactive inosine. HPLC experiments demonstrated that 2.5 U/ml of adenosine deaminase were sufficient to metabolize the 10 μ M exogenously added adenosine to

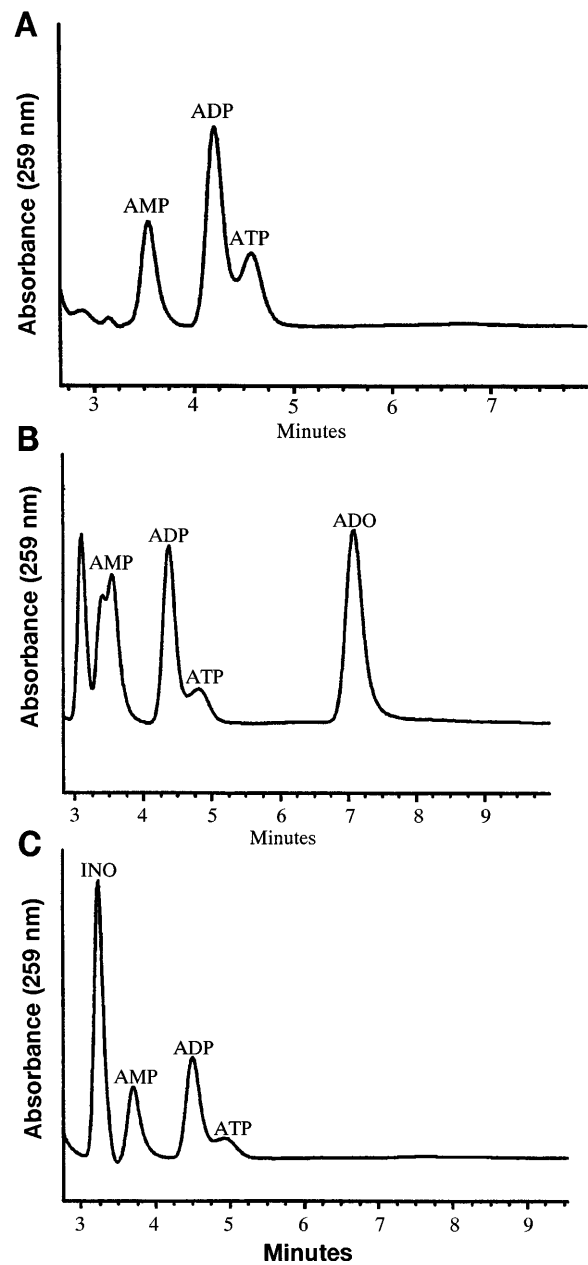


Fig. 1. A: as demonstrated by high-performance liquid chromatography (HPLC), concentration of adenine nucleotides in platelet-conditioned medium (PCM) was 1.72 μ M AMP, 5.12 μ M ADP, and 0.56 μ M ATP. Note absence of adenosine. B: adenosine deaminase metabolized 10 μ M adenosine (Ado) added exogenously to PCM within 30 min. Retention time for exogenously added Ado (10 μ M) was 7 min. C: Ado peak disappeared on addition of 2.5 U/ml of adenosine deaminase, and a prominent inosine (Ino) peak appeared. Peaks were identified by retention times of AMP, ADP, ATP, Ado, and Ino standards run at a concentration of 10 μ M. Peaks were quantitated by generating calibration curves with 3 concentrations of each standard (0- to 3-min solvent front not shown).

inosine within 30 min (Fig. 1, B and C). When platelets or PCM was incubated with endothelial cell monolayers, the clearance of ¹²⁵I-labeled albumin decreased by $> 30\%$ ($P < 0.05$; Fig. 2). Adenosine deaminase (2.5 U/ml) failed to block either the platelet- or PCM-induced decrease in albumin clearance, and adenosine deaminase by itself had no effect on albumin clearance

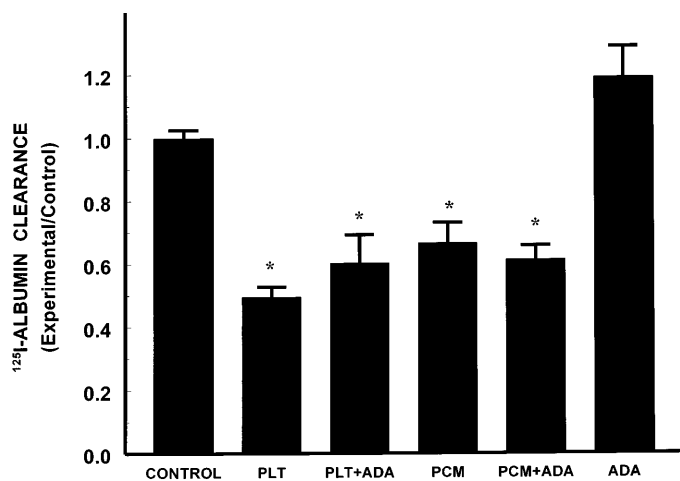


Fig. 2. Adenosine deaminase (ADA; 2.5 U/ml) did not prevent decrease in endothelial protein permeability induced by either platelets (PLT) or PCM. ADA (2.5 U/ml) alone had no effect on endothelial protein permeability. Control group was Dulbecco's modified Eagle's medium. Values are means \pm SE of ^{125}I -labeled albumin clearances normalized to control values for individual experiments; $n = 10$ cell monolayers/group. * $P < 0.01$ compared with control group.

(Fig. 2). This experiment was repeated with a different measurement of endothelial permeability, the resistive portion of electrical impedance. PCM increased endothelial electrical resistance across endothelial cell monolayers by $>20\%$, and adenosine deaminase (2.5 and 10 U/ml) did not prevent this increase (Fig. 3).

Binding of adenosine to its receptors on endothelial cells could explain the undetectable levels of adenosine in PCM as assessed by HPLC and could initiate a cell-signaling cascade via the A_2 -purinergic receptor. Therefore, the adenosine-receptor antagonist BW-A1433U83 ($K_D \sim 0.1 \mu\text{M}$) was used to block the binding of adenosine to the vascular endothelium. A 10-fold excess in concentration of the antagonist blocked the decrease in endothelial protein permeability induced by exogenously added adenosine (0.1 or 1 μM) but not the

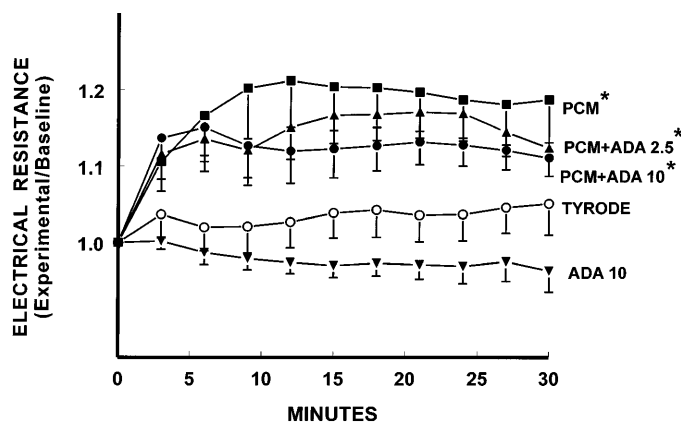


Fig. 3. ADA (2.5 and 10 U/ml) did not prevent increase in endothelial electrical resistance induced by PCM. ADA (10 U/ml) alone had no effect on electrical resistance. Tyrode buffer was vehicle control for all groups. Values are means \pm SE of electrical resistances normalized to 0 time values for individual cell monolayers; $n = 10$ cell monolayers/group. * $P < 0.05$ compared with Tyrode buffer control group at all time points in these 3 PCM groups except for 2.5-min PCM value.

decrease in endothelial protein permeability induced by platelets (Fig. 4A) or PCM (Fig. 4B).

To determine the effect of PCM with and without the presence of adenosine and adenine nucleotides, PCM was fractionated by a centricon microconcentrator or dialysis into <3 - and >3 -kDa fractions. Concentrations of AMP and ADP in control PCM were 1.25 and 4.82 μM , respectively. Adenosine and adenine nucleotides with molecular masses < 500 were filtered mostly into the <3 -kDa fraction where the concentration of AMP and ADP was 1.05 and 4.47 μM , respectively. In the >3 -kDa retentate fraction, AMP and ADP were significantly reduced to 0.20 and 0.35 μM , respectively. The retentate fraction (>3 kDa) decreased albumin clearance by $\sim 20\%$ ($P < 0.05$), whereas the filtrate fraction (<3 kDa) had no effect (Fig. 5A). Similar results were obtained on fractionation of the PCM by dialysis. The >3.5 -kDa fraction retained by dialysis, which also lacked detectable levels of adenosine, consistently de-

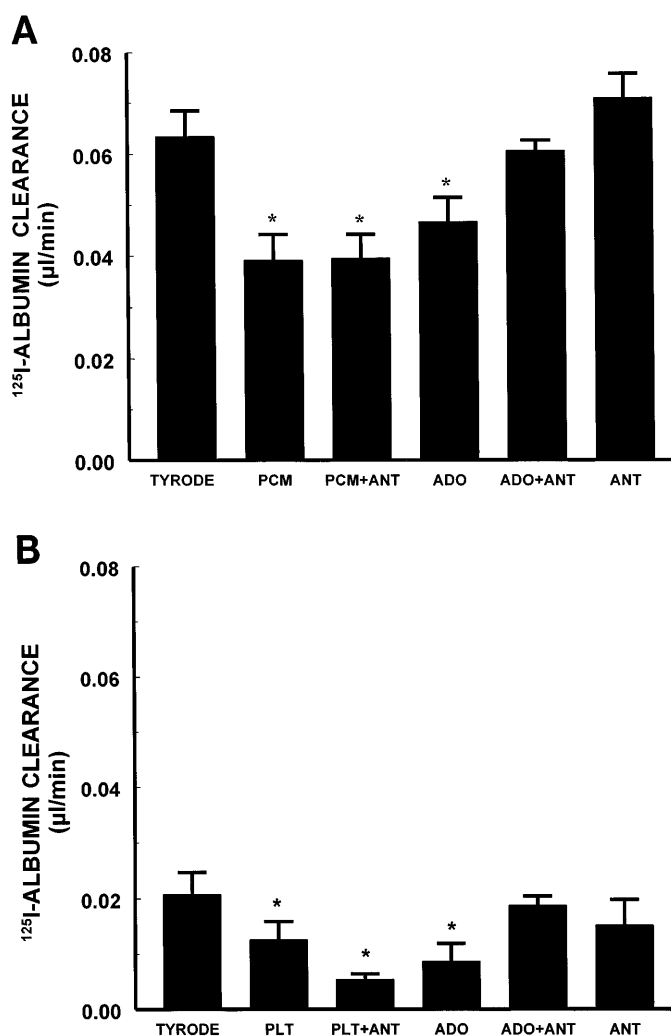


Fig. 4. Adenosine-receptor antagonist BW-A1433U83 (ANT; 10 μM) did not prevent permeability-decreasing activity of PCM (A) or platelets (B) but did block permeability response induced by ADO (1 μM). Values are means \pm SE; $n = 10$ cell monolayers/group. * $P < 0.05$ compared with control group.

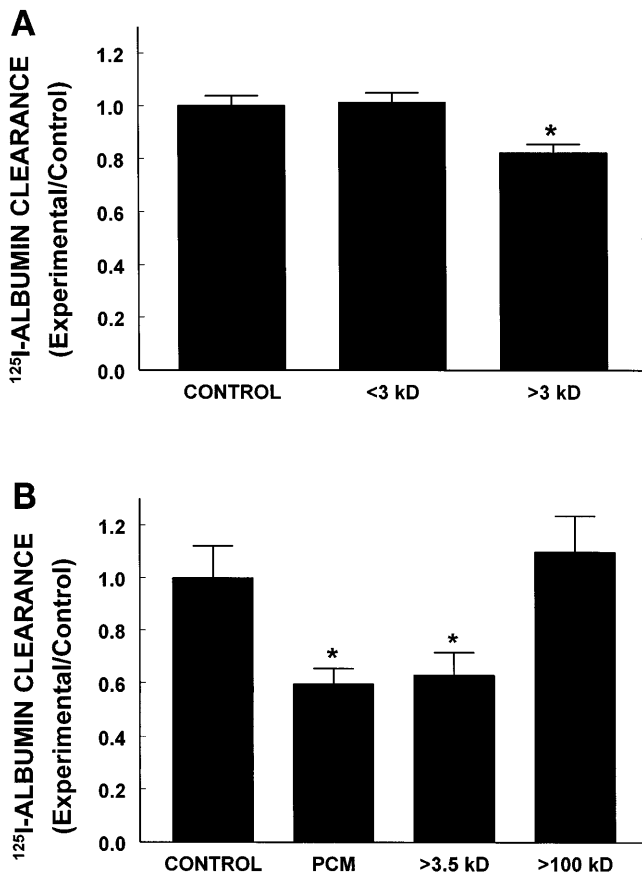


Fig. 5. *A*: fractionation of PCM by microconcentration produced a >3-kDa retentate that decreased endothelial protein permeability and a <3-kDa filtrate that had no permeability-decreasing activity. *B*: separation of PCM by dialysis resulted in a fraction > 3.5 kDa, retained by dialysis, that decreased endothelial protein permeability comparable to whole PCM and a fraction > 100 kDa, retained by dialysis, with no activity. Values are means \pm SE of ^{125}I -labeled albumin clearances normalized to control values for individual experiments; $n = 20$ cell monolayers/group. * $P < 0.05$ compared with control group (Tyrode buffer).

creased endothelial permeability ($P < 0.05$; Fig. 5*B*). The >12- to 14- and >50-kDa fractions retained by dialysis also decreased permeability (data not shown), whereas the fraction > 100 kDa had no effect on permeability (Fig. 5*B*).

Incubation of PCM with agarose-linked trypsin, which was subsequently removed by centrifugation, abolished the PCM-induced increase in endothelial electrical resistance (Fig. 6). Insoluble trypsin in Tyrode buffer induced the opposite response, a decrease in endothelial electrical resistance ($P < 0.05$). Removal of the insoluble trypsin in Tyrode buffer by centrifugation resulted in no change in endothelial electrical resistance. These latter findings suggest that the active factor in PCM is a protein.

SDS-PAGE gels of whole PCM showed five major bands from 15 to 200 kDa and many bands in between (Fig. 7). On treatment with trypsin, most bands were reduced in intensity or eliminated, although some of the bands remained unchanged.

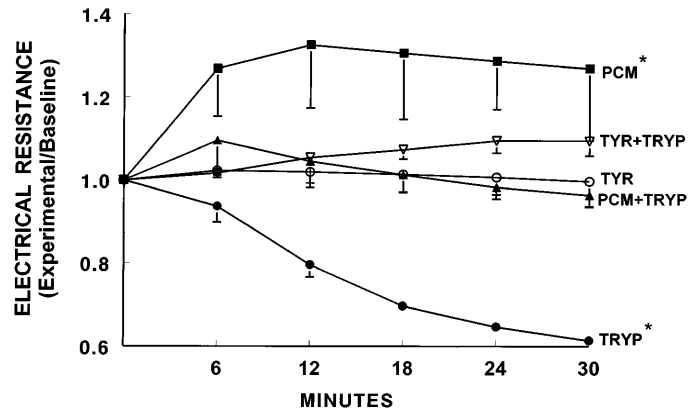


Fig. 6. Incubation of PCM for 10 min with trypsin cross-linked to beaded agarose (PCM+Tryp) inhibited increased endothelial electrical resistance induced by normal PCM. Insoluble trypsin in Tyrode buffer (Tryp) decreased electrical resistance. Insoluble trypsin added to Tyrode buffer (Tyr+Tryp) and then removed by centrifugation as well as Tyrode buffer (Tyr) alone had no effect on electrical resistance. Values are means \pm SE of electrical resistances normalized to 0 time values for individual cell monolayers; $n = 3$ cell monolayers/group. * $P < 0.05$ compared with 0-min values.

DISCUSSION

The objective of the present study was to determine whether adenosine is the primary factor responsible for the platelet-mediated decrease in endothelial protein

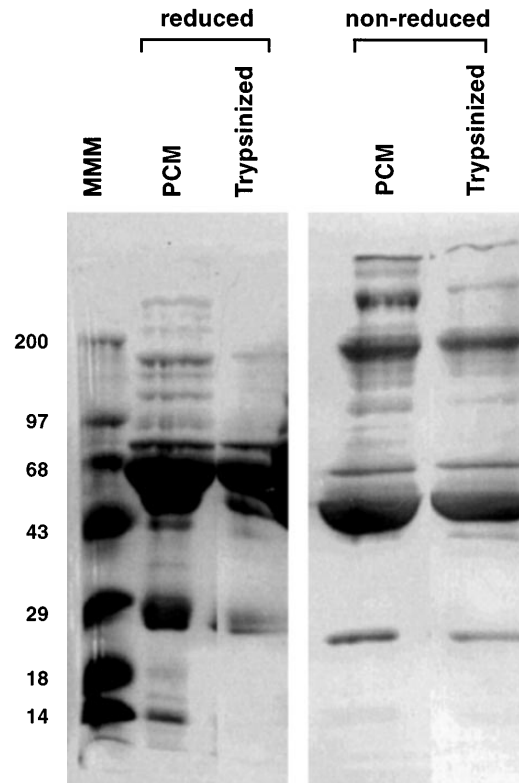


Fig. 7. Tryp-treated PCM showed bands similar to normal PCM, but most of the bands appeared with less intensity. In nonreduced gels, major bands in normal PCM were between 15 and 200 kDa, with a few bands > 200 kDa. Analytic SDS-polyacrylamide gel electrophoresis was done in 4–15% gradient polyacrylamide minilab gels. Gels were run from cathode (top) to anode (bottom) at 200 mV. Marker lane is not shown. MMM, molecular mass markers.

permeability *in vitro*. We correlated HPLC quantification of adenosine and adenine nucleotides in PCM with the measurements of ^{125}I -labeled albumin clearance and electrical resistance across endothelial cell monolayers. Adenosine was not detected in PCM, although micromolar concentrations of ATP, ADP, and AMP were present. Adenosine deaminase at a dose sufficient to metabolize 10 μM adenosine added exogenously to PCM did not block the PCM-induced decrease in endothelial permeability. The adenosine-receptor antagonist BW-A1433U83 blocked the decrease in permeability induced by adenosine but failed to block the decrease in permeability induced by PCM. A <3-kDa fraction of PCM that contained ADP and AMP did not decrease endothelial permeability, but a >3-kDa fraction with much reduced levels of these adenine nucleotides significantly decreased endothelial permeability. Furthermore, the permeability-decreasing activity of PCM was abolished by treatment with insoluble trypsin. We conclude from these findings that adenosine and its precursor adenine nucleotides are not the primary factors responsible for the platelet-induced decrease in endothelial permeability *in vitro*. Instead, a protein appears to possess this permeability-decreasing activity.

The literature is abundant with reports that thrombocytopenia in humans and animals is often associated with purpuric hemorrhages in skin and with tissue edema due to capillary leakage and that the lower the platelet count, the greater the frequency of hemorrhage. Transfusions with platelet-rich plasma have been shown to reverse the changes in vascular integrity in the skin of thrombocytopenic patients (15) as well as to reverse the changes in capillary permeability and edema in the perfused hindlimbs of frogs (9), in the erythrocyte content of thoracic duct lymph of thrombocytopenic dogs (23), in the erythrocyte content of lymph and vascular disappearance rate of ^{125}I -labeled albumin in the ear of thrombocytopenic rabbits (2), and in the increased permeability of fluorescein isothiocyanate-labeled albumin in the coronary microvasculature of thrombocytopenic rats (18). Gimbrone et al. (12) observed that gross purpura, endothelial gaps, and increased leakage of ^{51}Cr -erythrocytes and ^{125}I -labeled albumin occur in an isolated lobe of the thyroid in dogs perfused with platelet-poor plasma but not in the other isolated lobe perfused with platelet-rich plasma. Lo et al. (17) provided the first evidence of a protein permeability defect in the pulmonary vasculature of thrombocytopenic animals. Sheep chronically depleted of platelets for 3 days with antiplatelet antibodies develop the characteristic petechial hemorrhages seen in thrombocytopenic patients, an increased endothelial protein permeability in the lung, and severe pulmonary edema at a left atrial pressure of 20 mmHg. These effects are rapidly reversed by transfusion with platelet-rich plasma. Shepro et al. (25) suggested that platelets continually release a humoral factor from granules that decreases endothelial permeability. In support of this concept, it has been shown that PCM and platelet lysate also possess permeability-decreasing activity (22, 24).

Subsequent experiments have focused on serotonin, norepinephrine, cyclooxygenase metabolites, and adenosine as potential factors responsible for the permeability-decreasing activity of platelets (13, 24, 25). Shepard et al. (24) found that this activity of platelets is not inhibited by propranolol, a β -adrenergic-receptor antagonist; ketanserin, a serotonin-receptor antagonist; or aspirin, a cyclooxygenase inhibitor. Adenosine, a potent vasodilator, is known to decrease endothelial protein permeability *in vitro* and *in vivo* (1, 14, 22). This response is mediated primarily by A_2 -purinergic receptors on endothelial cells that, when activated, increase the intracellular level of adenosine 3',5'-cyclic monophosphate (1, 14). Our results show that PCM contained AMP, ADP, and ATP but no detectable levels of adenosine (sensitivity $\geq 0.1 \mu\text{M}$). However, on incubation of PCM with endothelial cells, adenosine can be generated mainly via sequential dephosphorylation of adenine nucleotides. Human platelets contain high concentrations of adenine nucleotides ($\sim 10 \text{ mM}$) distributed between the metabolic (cytoplasmic) and storage (dense granules) pools. Coade and Pearson (5) showed that ATP and ADP released from platelets can be rapidly converted to adenosine by endothelial ectonucleotidases, which results in transient local concentrations of adenosine as high as 100 μM . When PCM was incubated with endothelial cells in the present study, the amount of ADP and ATP as detected by HPLC was reduced and AMP was increased, but no adenosine was detected (data not shown). This measurement shows that even though adenine nucleotides in the PCM were gradually dephosphorylated, no detectable amount of adenosine was produced. Possible explanations for the undetected adenosine are the level of sensitivity of the HPLC assay ($\geq 0.1 \mu\text{M}$), rapid metabolism by adenosine deaminase to inosine (although no inosine peak was detected), conversion back to AMP by adenosine kinase, uptake of adenosine in the endothelial cells by facilitated transport, low levels of 5'-nucleotidase in the endothelial cells, and binding of adenosine to its receptors on the cell. It is unlikely that receptor-bound adenosine induced the functional response in the present study because the adenosine-receptor antagonist BW-A1433U83 did not block either the platelet- or PCM-induced decrease in permeability, although it did block the decreased permeability induced by exogenously applied adenosine.

The role of adenosine as the primary factor responsible for the platelet-induced decrease in endothelial permeability is controversial. Initial evidence for the role of platelet-derived adenosine was put forth by Paty et al. (22) based on their experiments with endothelial cell monolayers and isolated, perfused guinea pig lungs. They demonstrated that platelets, PCM, and 0.1 μM adenosine all decreased endothelial permeability and that 100 U/ml of adenosine deaminase blocked the permeability-decreasing effect of platelets and PCM. In contrast, Haselton and Alexander (13) were not able to inhibit the platelet response using 2.5 U/ml of adenosine deaminase. Various factors could contribute to these different outcomes by these two groups. First, the

doses of adenosine deaminase used by the two groups were vastly different: 100 U of adenosine deaminase/ml of PCM by Paty et al. (22) compared with 2.5 U/ml of PCM by Haselton and Alexander (13). Second, different endothelial cells were used. Paty et al. (22) used endothelial cells from the bovine pulmonary artery, and Haselton and Alexander (13) used bovine adult and fetal aortic endothelial cells. Third, different techniques were used to measure changes in endothelial permeability. Paty et al. (22) used a modified Boyden chamber consisting of a luminal chamber floating in an abluminal chamber, allowing for the measurement of albumin clearance in the absence of hydrostatic and oncotic pressure gradients. Haselton and Alexander (13) used a model consisting of a chromatographic cell column filled with microcarrier beads covered with endothelial cells. This model, which is like a gel filtration column, measures the permeability of small-molecular-mass tracers (<2 kDa). In the present study, we used the same cell line and permeability model that was used by Paty et al. (22).

Because different doses of adenosine deaminase were used in the above two studies, it was important to determine in the present study the dose of adenosine deaminase sufficient to metabolize adenosine in the PCM to inosine. We were unable to detect any adenosine in the PCM, so we added 10 μ M adenosine under the assumption that the dose of adenosine deaminase sufficient to metabolize 10 μ M adenosine should also be sufficient to metabolize any undetectable level of adenosine in the PCM. Adenosine deaminase at 2.5 U/ml completely metabolized the 10 μ M adenosine added exogenously to the PCM within 30 min but did not block the decrease in endothelial permeability, as measured by albumin clearance and electrical resistance, induced by either whole platelets or PCM.

In an attempt to study the function of PCM in the absence of adenosine or its precursor adenine nucleotides, PCM was separated by microconcentration or dialysis into <3- and >3-kDa fractions. The <3-kDa fraction of PCM with an adenine nucleotide profile identical to that of intact PCM had no effect on monolayer permeability, whereas the fraction > 3 kDa, which had much reduced levels of adenine nucleotides, retained the ability to decrease permeability. Dialysis experiments revealed that the permeability-decreasing activity of PCM resided between 3.5 and 100 kDa. Haselton and Alexander (13) fractionated the PCM using centricon microconcentration (100-kDa molecular-mass cutoff) into >100- and <100-kDa fractions and determined that a >100-kDa protein was responsible for the platelet effect. This estimate of molecular mass was based on spin filter separation that we have found to be unreliable as demonstrated by SDS-PAGE gels that show protein bands < 100 kDa in the retained fraction from a microconcentrator with a 100-kDa molecular-mass cutoff. We have made similar observations with microconcentrators with 30-, 50-, and 100-kDa molecular-mass cutoffs. Unfortunately, the same is true about dialysis with membranes with various size molecular-mass cutoffs. Nevertheless, the present study

and that of Haselton and Alexander (13) agree that the permeability-decreasing activity of PCM resides in a fraction > 3 kDa that does not contain adenosine and the adenine nucleotides.

Further evidence against adenosine is that the active factor appears to be a protein because the activity of PCM is trypsin sensitive. Haselton and Alexander (13) found the activity of PCM to be sensitive to soluble trypsin that was neutralized with soybean inhibitor. Because soluble trypsin induces the exact opposite effect on endothelial permeability as PCM, insoluble trypsin, cross-linked to beaded agarose, was used in the present study. In Tyrode buffer, insoluble trypsin alone decreased endothelial electrical resistance, but when removed by centrifugation, there was no effect on electrical resistance. When incubated with PCM and then removed by centrifugation, insoluble trypsin abolished the activity of PCM. It is likely that the protein is very sensitive to trypsin because the activity of PCM was abolished after 10 min of trypsin treatment, yet SDS-PAGE gels of the trypsin-treated PCM showed that many of the bands remained intact, although diminished in concentration.

The notion that the active factor in platelets is a protein is not novel since Wilbrandt et al. (30) reported in 1956 that a protein fraction extracted from platelets reduces capillary permeability in the rat hindlimb. That the activity in PCM can be precipitated by 60% saturated ammonium sulfate (unpublished observations) provides evidence that the factor is not a small protein because this fractionation procedure precipitates large, charged molecules.

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