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Platelet activation through interaction with hemodialysis membranes induces neutrophils to produce reactive oxygen species

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Abstract: The intradialytic activation of leukocytes is one of the major causes of hemodialysis-associated complications. During hemodialysis, the formation of microaggregates consisting of platelets and neutrophils has been observed to accompany the production of reactive oxygen species (ROS) by leukocytes. In this study, we investigated the interaction of platelets and neutrophils with hemodialysis membranes *in vitro* to elucidate the mechanism underlying microaggregate formation and its relevance to leukocyte activation. The production of ROS in neutrophils was induced by the coincubation of neutrophils with polysulfone (PS) membranes, and was increased when platelets were present in the neutrophil suspension. Neutrophils that were incubated with polymethylmethacrylate (PMMA) membranes in the presence of platelets also produced significant levels of ROS, suggesting that the presence of platelets augmented ROS production in neutrophils. Platelets adhered more firmly to hydrophobic membranes such as PS and PMMA membranes than to hydrophilic membranes, such as those composed of regenerated cellulose (RC) or ethylene vinylalcohol copolymer (EVAL). The adhesion of platelets to dialysis membranes composed of different materials was correlated with those membranes' ability to induce platelet activation as assessed by the cell surface expression of P-selectin. Moreover, coincubation of neutrophils with platelets that had been treated with hydrophobic membranes induced a higher level of superoxide anion relative to those treated with hydrophilic membranes in association with the P-selectin-mediated microaggregate formation. These results suggest that platelets activated through interaction with hemodialysis membranes stimulate neutrophils to produce ROS via P-selectin-mediated adhesion, and that this property of adhesion to platelets is critical for the biocompatibility of hemodialysis membranes. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 77A: 294–303, 2006

Key words: hemodialysis; reactive oxygen species; platelet; neutrophil; cell adhesion

INTRODUCTION

The interaction between blood components and dialysis membranes is a key factor in determining the biocompatibility of hemodialyzers. The inadequate activation of leukocytes during hemodialysis is thought to cause acute and chronic complications associated with hemodialysis therapy, such as leukopenia, fever, hypotension, hypertension, immunologic dysfunction, and atherosclerosis.[1–3] The activation of complement through its interaction with dialysis membranes is reportedly a major cause of leukocyte activation.[4] In addition, the intercellular adhesion of blood cells has also been investigated since the formation of platelet-leukocyte microaggregates was observed during hemodialysis.[5] The adhesion between platelets and neutrophils was reported to induce the mutual activation of these cells, such as increased expression of integrin CD11b on neutrophils and platelet activation stimulated by neutrophil-derived platelet-activating factor and eicosanoids.[6–8] Recently, Bonomini and coworkers reported that the production of reactive oxygen species (ROS) was induced in conjunction with the formation of platelet–neutrophil microaggregates during hemodialysis, and that the process involved the interaction between these blood cells and hemodialysis membranes.[9–11] Activated platelets express various cell adhesion molecules on their surface in association with degranulation. P-selectin, a member of the selectin family of adhesion molecules, is known to be expressed on activated endothelial cells and activated platelets.[12] In resting platelets, P-selectin is stored in α-granules and translocated onto the cell surface upon cellular activation. This adhesion molecule recognizes the carbohydrate-containing counter-ligands on leukocytes and plays a role in leukocyte recruitment to

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hemorrhagic sites.[13] We previously reported that the adhesion of activated platelets to leukocytes, mediated through the interaction between P-selectin and its counter-ligands, is capable of stimulating neutrophils and monocytes to produce ROS.[7,14] The ROS produced by leukocytes causes vascular inflammation leading to hemodialysis-associated complications. Therefore, the formation of platelet–neutrophil microaggregates should be controlled in hemodialytic therapy. The microaggregate formation depends on the materials composing the dialysis membranes, suggesting the importance of the interaction between blood cells and hemodialysis membranes. In the present study, we examined *in vitro* the adhesive properties of hemodialysis membranes with distinct materials for platelets and neutrophils to elucidate the mechanism underlying the microaggregate formation and its relevance to leukocyte activation. The results showed that the adhesion of platelets to hemodialysis membranes was of primary importance to the ROS production process.

MATERIALS AND METHODS

Reagents and hemodialysis membranes

Adenosine diphosphate (ADP) and Dextran 200,000 were purchased from Wako Pure Chemical Industries (Osaka, Japan). 2′,7′-Bis(carboxyethyl)carboxyfluorescein tetraacetoxymethyl ester (BCECF-AM) was from Dojindo Laboratories (Kumamoto, Japan). 2′,7′-dichlorodihydrofluorescein diacetate (DCFH–DA), Triton X-100, cytochrome *c*, and superoxide dismutase were products of Sigma Chemical (St. Louis, MO). Heparin was from Mochida Pharmaceutical (Tokyo, Japan). Ficoll-Paque was from Amersham Biosciences (Uppsala, Sweden). GRGDS (Gly-Arg-Gly-Asp-Ser) peptide and GPRP peptide (Gly-Pro-Arg-Pro) peptide were supplied by Bachem (Bubendorf, Switzerland). Anti-P-selectin blocking antibody (2T60, IgG) was kindly donated by Dr. Kenjiro Taoue (The Tokyo Metropolitan Institute of Medical science).[7] Anti-P-selectin nonblocking antibody (2D7, IgG) was prepared in our laboratory.[15] Anti-PSGL-1 antibody (PL1, IgG) was purchased from Serotec Ltd. (Oxford, UK). Anti-sialyl LeX antibody (KM93, IgM) was a generous donation from Dr. Nobuo Hanai (Kyowa Hakko Kogyo, Tokyo, Japan).[14] Alexa Fluor 488-conjugated goat anti-mouse IgG antibody, Alexa Fluor 555-conjugated streptavidin, and TO-PRO-3 iodide were purchased from Molecular Probes (Eugene, OR). Biotinylated anti-mouse IgM antibody was from ICN Pharmaceuticals (Aurora, OH). Horseradish peroxidase-conjugated goat anti-mouse IgG antibody was from KPL (Gaithersburg, MD). TMB (3,3′,5,5′-tetramethylbenzidine) reagent was from PharMingen (San Diego, CA). The following hemodialysis membranes (hollow fibers) were used: polysulfone (PS, internal diameter = 230 nm, external diameter = 280 nm), polymethylmethacrylate (PMMA, internal diameter = 200 nm, external diameter = 240 nm), ethylene vinylalcohol copolymer (EVAL, internal diameter = 200 nm, external diameter = 260 nm), and regenerated cellulose (RC, internal diameter = 200 nm, external diameter = 230 nm). Hollow fibers were removed from commercially available dialyzers, cut into small pieces 1–2 mm in length, under sterile conditions, and stored in sterile saline until use.

Platelets and neutrophils

Heparinized blood from healthy volunteers was centrifuged at 800 rpm for 10 min to obtain platelet-rich plasma (PRP). The pelleted cells were mixed with an equal volume of 3% dextran 200,000/saline to sediment most of the erythrocytes. After standing for 30 min, the supernatant was subjected to Ficoll-Paque density gradient centrifugation. Neutrophils were purified from the pelleted cells by hypotonic lysis of the remaining erythrocytes. The purity of the neutrophils was greater than 95% as assessed by May-Grünwald-Giemsa staining.

Measurement of intracellular hydrogen peroxide production

The intracellular hydrogen peroxide production by neutrophils, after coincubation with dialysis membranes, was determined by a DCFH oxidation assay as described by Bass et al.[16] Briefly, neutrophils suspended in autologous plasma or PRP (200 μL, at a cell density of 1×10^7 cells/mL) were incubated with pieces of hemodialysis membranes (derived from 34 cm of hollow fibers) for 30 min, with continuous agitation. DCFH-DA (0.1 m*M* at a final concentration) was added to the neutrophil suspension, and the mixture was incubated at 37°C for 30 min. The oxidized form of DCFH was measured by a flow cytometer (FACS Calibur, BD Biosciences, San Diego, CA). Neutrophils were distinguished from platelets in a forward-scatter versus side-scatter plot. Data were collected for 10,000 cells gated for neutrophils.

Adhesion of platelets and neutrophils to hemodialysis membranes

Platelets suspended in plasma were fluorescently labeled with BCECF-AM (0.05 m*M*) for 30 min at 37°C. After the addition of 1/10 volume of ACD solution (0.08*M* citric acid, 0.12*M* sodium citrate, 0.11*M* D-glucose), BCECF-labeled platelets were recovered by centrifugation at 1,000 × *g* for 5 min and resuspended in an original volume of autologous plasma or heparinized blood. Pieces of dialysis membranes (derived from 17-cm length of hollow fibers) were added to 200 μL of the platelet suspension, and the suspension was incubated for 1 h at 37°C, with continuous agitation (120 min^{-1}). After unadhered platelets were removed by washing five times with PBS, the platelets adhering to the dialysis membranes were lysed with 100 μL of 1% Triton X-100. The number of adhered platelets was estimated by BCECF fluorescence associated with adhered platelets using a fluorescence spectrophotometer (Model F-4010, Hitachi Ltd., To-

kyo, Japan; Ex = 490 nm, Em = 520 nm). Neutrophils were also labeled with BCECF-AM (0.01 m*M*) in the same procedure as for the platelets, and were resuspended in plasma at a cell density of 1×10^7 cells/mL. The labeled neutrophils were incubated with dialysis membranes, and the cell adhesion was determined by the fluorescence intensity associated with the cells adhering to membranes as described above.

Measurement of P-selectin on the platelet surface by cell-based ELISA

PRP (200 μL) was incubated with pieces of dialysis membranes (derived from 68-cm length of hollow fibers) at 37°C for 1 h with continuous agitation, and platelets were fixed with 1 mL of 1% formaldehyde-PBS for 10 min at room temperature. The fixed platelets were washed with PBS and incubated with PBS containing 4% BlockAce™ (a milk protein-based blocking reagent, Dainippon Pharmaceutical, Osaka, Japan) for 30 min at room temperature. Platelets were successively treated with anti-P-selectin antibody (2D7, 5 μg/mL in 4% BlockAce™/PBS) for 30 min, and with horseradish peroxidase-conjugated anti-mouse IgG antibody. After being washed twice with PBS, platelets were resuspended in 200 μL of TMB reagent and incubated for 5 min, and then 100 μL of 1*M* phosphoric acid was added to the suspension. The supernatant obtained after centrifugation of the mixture was colorimetrically assayed by using a microplate reader (A_{450}–A_{620}). The expression of P-selectin on the platelet surface was estimated after normalization with the total protein content of each sample.

Measurement of cell surface P-selectin by flow cytometry

Formaldehyde-fixed platelets prepared by the same procedures as described above were stained with a combination of anti-P-selectin monoclonal antibody (2D7) and Alexa Fluor 488-conjugated secondary antibody, and were subjected to flow cytometric analysis.

Confocal laser-scanning microscopic observation of platelet–neutrophil microaggregates

PRP (200 μL) was incubated with dialysis membranes at 37°C for 1 h, and platelets were recovered from PRP by centrifugation at 1,000 × *g* for 5 min. The pelleted platelets were resuspended in 100 μL of PBS, mixed with 100 μL of neutrophil suspension (2×10^7cells/mL), and incubated at 37°C for 30 min. After the cells were fixed with 1% formaldehyde, they were washed with PBS and stained with anti-P-selectin antibody (2D7) plus Alexa Fluor 488-conjugated anti-IgG antibody for platelets and with a combination of anti-sialyl LeX antibody (KM93), biotinylated anti-mouse IgM antibody, and Alexa Fluor 555-conjugated streptavidin for neutrophils. The nuclei were stained with TO-PRO-3 iodide (5 μ*M*). The fluorescently labeled cells were observed with a confocal laser-scanning microscope (Radians 2100, Bio-Rad Laboratories, Hercules, CA).

Flow cytometric analysis of the platelet–neutrophil microaggregate formation

The platelet–neutrophil microaggregate formation was analyzed by flow cytometry essentially as described previously.[14] Briefly, BCECF-labeled platelets in autologous plasma (300 μL) were incubated with pieces of PS membranes at 37°C for 30 min as described above. An aliquot (50 μL) was mixed with neutrophils (4×10^6 cells/mL, 50 μL) and incubated for 20 min at 37°C or 4°C with continuous agitation in the presence or absence of antibodies (20 μg/mL), EDTA (5 m*M*), or adhesion inhibitory peptides (2 m*M*). The mixture was then diluted with 3 mL of PBS and subjected to flow cytometric analysis. Neutrophils were gated on a forward-scatter versus side-scatter plot. The platelet–neutrophil microaggregates were quantified by counting neutrophils positive for fluorescence associated with BCECF-labeled platelets. Data were collected for 2,000 cells gated for neutrophils.

Measurement of superoxide anion produced by neutrophils

Production of superoxide anion by neutrophils that had been incubated with platelets was determined by the cytochrome *c* reduction assay.[17] The platelets were coincubated with hemodialysis membranes as described above. Platelet suspension (50 μL) was mixed with neutrophil suspension (1×10^6 cells, 50 μL) and cytochrome *c* solution (0.1 m*M*, 100 μL), and the mixture was incubated at 37°C for 90 min with gentle agitation. After centrifugation of the mixture at 5,000 rpm for 10 min, the supernatant was measured for A_{540}, A_{550}, and A_{560}. The change in A_{550} caused by the reduction of cytochrome *c* was calculated by the following formula: $\Delta A = A_{550} - (A_{540} + A_{560})/2$. The contribution of superoxide anion was determined by subtracting the differences in ΔA values measured in the absence or presence of superoxide dismutase (300 U/mL).[7]

RESULTS

ROS production by neutrophils after coincubation with hemodialysis membranes

We first examined the production of ROS by neutrophils when they were incubated with four types of hemodialysis membranes consisting of distinct materials. The intracellular hydroperoxide production was measured by the DCFH-DA oxidation assay using flow cytometry.[16] To assess the involvement of platelets in neutrophil ROS production, we analyzed the ROS production in the presence or absence of platelets

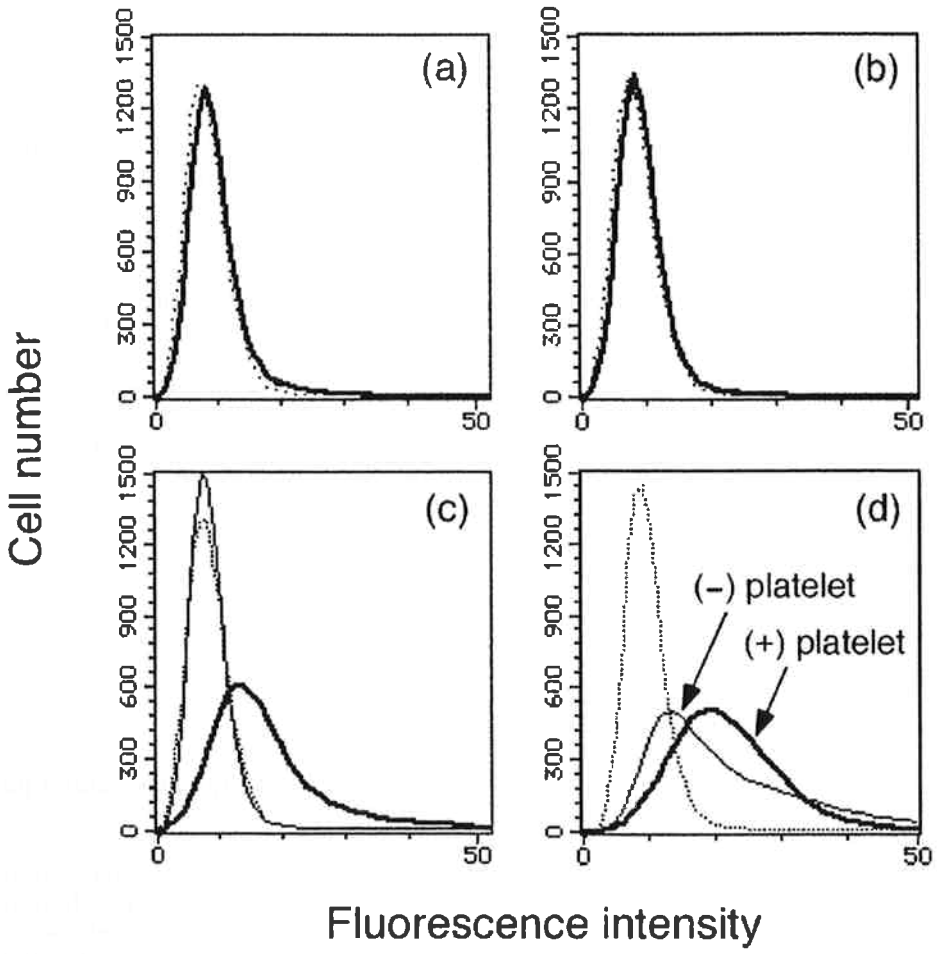

Figure 1. Production of intracellular hydroperoxide in neutrophils incubated with hemodialysis membranes in the presence or absence of platelets. Human neutrophils were suspended in plasma (−platelets) or platelet-rich plasma (+platelets) and incubated with hemodialysis membranes for 30 min. After the addition of DCFH-DA (0.1 m*M*) to the suspension, the mixture was incubated for 30 min. The generation of the oxidized form of DCFH was assayed by flow cytometry. *Dotted line*, neutrophils incubated without dialysis membranes; *thin line*, neutrophils incubated with dialysis membranes in the absence of platelets; *thick line*, neutrophils incubated with dialysis membranes in the presence of platelets. (a), RC membranes; (b), EVAL membranes; (c), PMMA membranes; (d), PS membranes. Representative data of three experiments are shown.

(Fig. 1). When neutrophils were incubated with PS membranes in the absence of platelets, the fluorescence profile was slightly shifted as compared to neutrophils incubated without hemodialysis membranes [Fig. 1(d)], indicating that coincubation with PS membranes induced the generation of ROS. ROS production was augmented when neutrophils were incubated with the same membranes in the presence of platelets. Neutrophils that had been incubated with PMMA membranes in the presence of platelets produced ROS at significant levels, whereas almost no ROS production was detected in neutrophils incubated in the absence of platelets [Fig. 1(c)]. In contrast, neutrophils incubated with RC or EVAL membranes did not produce detectable levels of ROS, irrespective of the presence or absence of platelets [Fig. 1(a,b)]. These results indicated that the ROS production in neutrophils incubated with hemodialysis membranes depends on the materials composing the hollow fibers of the hemodialyzer, and that platelets were greatly involved in these processes.

Adhesion of platelets and neutrophils to hemodialysis membranes

As the results of the ROS production assay suggested the importance of the interaction of platelets and neutrophils with membranes, we next examined the adhesion of these cells to hemodialysis membranes. Platelets suspended in autologous plasma adhered more firmly to PS and PMMA membranes than to RC or EVAL membranes [Fig. 2(a)]. Approximately 4% of platelets added to the incubation mixture adhered to PS membranes under the conditions used. Similar results were obtained when labeled platelets were suspended in whole blood instead of plasma

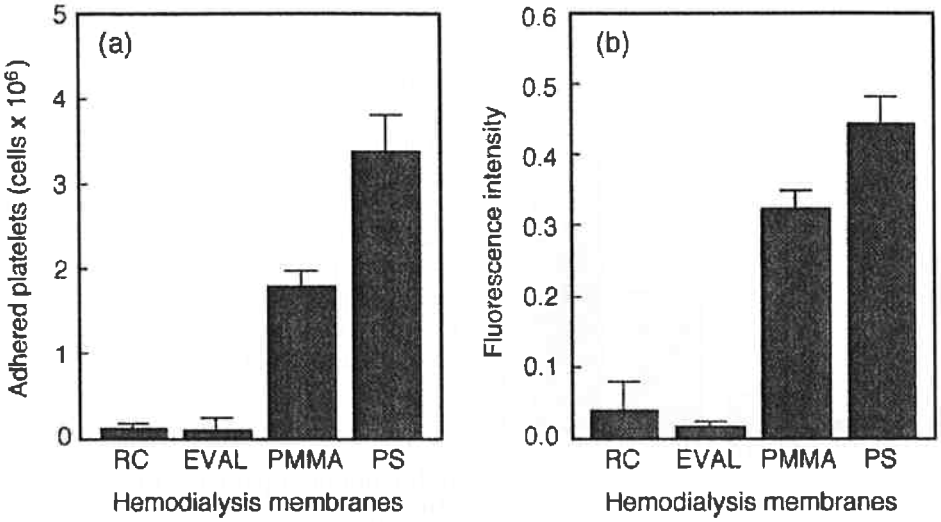

Figure 2. Adhesion of platelets to hemodialysis membranes. BCECF-labeled platelets were suspended in autologous plasma (a) or whole blood (b) and incubated with dialysis membranes at 37°C for 1 h. After unadhered platelets were removed by washing with PBS, platelets adhering to the membranes were lyzed with 1% Triton X-100, and the fluorescence of BCECF in the lysate was measured with a fluorescence spectrophotometer (Ex = 490 nm, Em = 520 nm).

[Fig. 2(b)]. Platelet adhesion appears to depend on the hydrophilic or hydrophobic properties of the materials composing the hemodialysis membranes. That is, platelets preferentially adhered to hydrophobic surfaces rather than to hydrophilic surfaces. This conjecture was supported by the result that platelets adhered more to unmodified PS membranes than to PS membranes that had been modified to increase surface hydrophilicity by blending them with polyvinylpyrrolidone (data not shown). On the other hand, no significant difference in the adhesion of neutrophils to hemodialysis membranes was observed among the different membranes, except that slightly more neutrophils adhered to the PS membranes than to the others (Fig. 3). These results suggest that platelet adhesive properties are correlated with the inducibility of ROS production in neutrophils in the presence of platelets.

Induction of P-selectin expression on platelet surface

P-selectin is known to be expressed on activated platelets and to mediate platelet–neutrophil adhesion.[13] We examined the expression of P-selectin on platelets after their coincubation with hemodialysis membranes by the cell-based ELISA method. The incubation of PRP with PMMA or PS membranes was found to induce P-selectin expression on the platelet surface, whereas no significant expression of P-selectin was detected when PRP was incubated with EVAL membranes (Fig. 4). The P-selectin expression was also analyzed by flow cytometry (Fig. 5). The percentages of P-selectin-positive platelets after coincubation with the four types of hemodialysis membranes were estimated as follows: negative control (incubation without hemodialysis membranes), 0.23%; EVAL membranes, 0.58%; PMMA membranes, 1.85%; PS membranes, 7.55%. The results from the flow cytometric analysis were in good agreement with those from the cell-based ELISA. The expression of P-selectin was induced most by PS membranes, followed by PMMA, and these results paralleled the adhesive properties of the membranes for platelets shown in Figure 2.

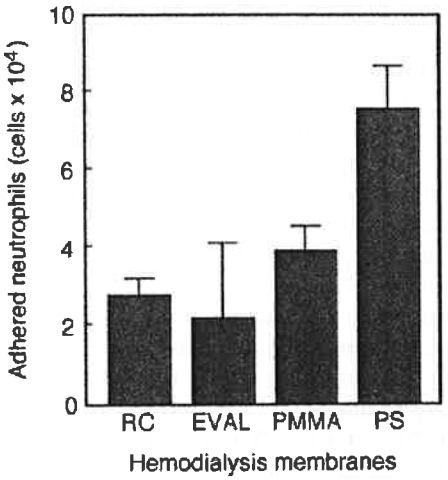

Figure 3. Adhesion of neutrophils to hemodialysis membranes. BCECF-labeled neutrophils were suspended in autologous plasma (1×10^7 cells/mL) and then were incubated with dialysis membranes at 37°C for 1 h. After unadhered neutrophils were removed by washing with PBS, neutrophils adhering to the membranes were lyzed with 1% Triton X-100, and the fluorescence associated with the adhered cells was measured with a fluorescence spectrophotometer (Ex = 490 nm, Em = 520 nm).

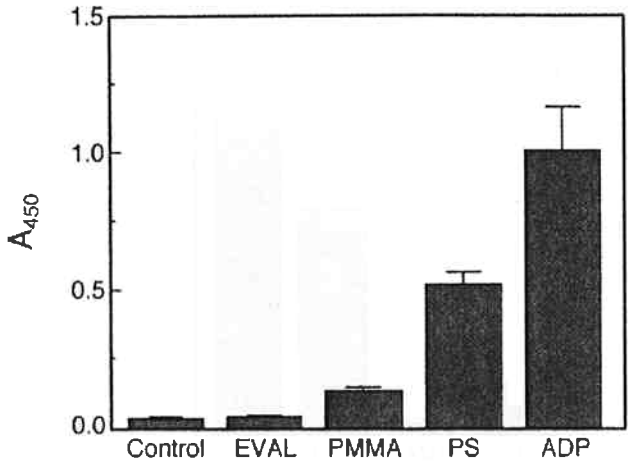

Figure 4. Expression of P-selectin on the surface of platelets after coincubation with hemodialysis membranes. PRP was incubated with hemodialysis membranes at 37°C for 1 h. Platelets were fixed with 1% formaldehyde-PBS at room temperature for 10 min, and the expression of P-selectin on the platelet surface was measured by cell-based ELISA, as described in Materials and Methods. Platelets were activated with ADP (20 μ*M*) for a positive control.

Formation of a platelet–neutrophil microaggregate

We next examined whether or not platelet–neutrophil microaggregates were formed *in vitro* when these cells were incubated with hemodialysis membranes, as was demonstrated during hemodialysis.[5,11] Immunofluorescence observation indicated that platelets pretreated with PS or PMMA membranes expressed P-selectin on their surface and formed a complex with neutrophils (Fig. 6). In addition to the heterotypic complex of platelets and neutrophils, a homotypic microaggregate of platelets was also observed. To evaluate P-selectin's contribution to microaggregate formation, the effects of specific monoclonal antibodies against P-selectin and its counter-ligand, PSGL-1 (P-selectin glycoprotein ligand-1), were examined by utilizing flow cytometry, in which neutrophils positive for fluorescence due to a physical association with BCECF-labeled platelets were measured [Fig. 7(a)]. Approximately 30% of the neutrophils were detected in the fluorescence-positive region after incubation with PS membrane-treated platelets in the absence of antibody. The microaggregate formation was almost completely inhibited by anti-P-selectin blocking antibody (2T60) and anti-PSGL-1 antibody (PL1) but not by anti-P-selectin non-blocking antibody (2D7) [Fig. 7(b)]. The inhibitory effects of 2T60 (20 μg/mL) and PL1 (20 μg/mL) on the microaggregate formation were comparable to that of 5 m*M* EDTA. We next examined whether or not the integrin-mediated interaction is involved in microaggregate formation, since the platelet integrin αIIbβ3 (GPIIb/IIIa) was reported to play a role in the adhesion of activated platelets to neutrophils via fibrinogen.[18] However, neither the integrin-recognizing GRGDS peptide nor the fibrin polymerization-inhibiting GPRP peptide inhibited PS membrane-induced platelet–neutrophil microaggregate formation [Fig. 7(c)]. Microaggregates also formed when these cells were incubated at 4°C. These results indicated that the interaction of platelets with hemodialysis membranes induced platelet activation and the subsequent formation of microaggregates consisting of platelets and neutrophils, and that the microaggregate formation was mediated predominantly by the interaction between P-selectin on activated platelets and PSGL-1 on neutrophils.

Induction of superoxide production by neutrophils after coincubation with platelets activated by hemodialysis membranes

We previously reported that the adhesion of activated platelets stimulated neutrophils to produce ROS through the interaction between P-selectin on platelets and its counter-ligands on neutrophils.[7,19] We therefore examined whether or not platelets treated with hemodialysis membranes induced neutrophils to generate ROS. The experimental conditions for the treatment of platelets with hemodialysis membranes were identical to those in the observation of platelet–neutrophil microaggregates shown in Figure 6. Platelets pretreated with PMMA membranes induced neutrophils to produce superoxide anion, while those treated with EVAL membranes did not induce neutrophils to generate a significant level of superoxide anion (Fig. 8). These results are consistent with those from the ROS production assay assessed by flow cytometry, in which a mixture of platelets and neutrophils was incubated with hemodialysis membranes [Fig. 1(b,c)]. Therefore, it is likely that the contact between platelets and hemodialysis membranes is of primary importance in the stimulation of neutrophils to produce ROS.

DISCUSSION

In the present study, we analyzed the interaction of platelets and neutrophils with hemodialysis membranes *in vitro*, and demonstrated that the membranes induced platelet activation, resulting in the formation of platelet–neutrophil microaggregates via P-selectin-mediated adhesion. The results also suggested that the adhesion of activated platelets to neutrophils caused the neutrophils to produce ROS. The activation of platelets depended on the materials composing the hollow fibers of the hemodialyzer. Hydrophobic membranes, such as those composed of PS or PMMA, possessed higher levels of activity for platelet adhe-

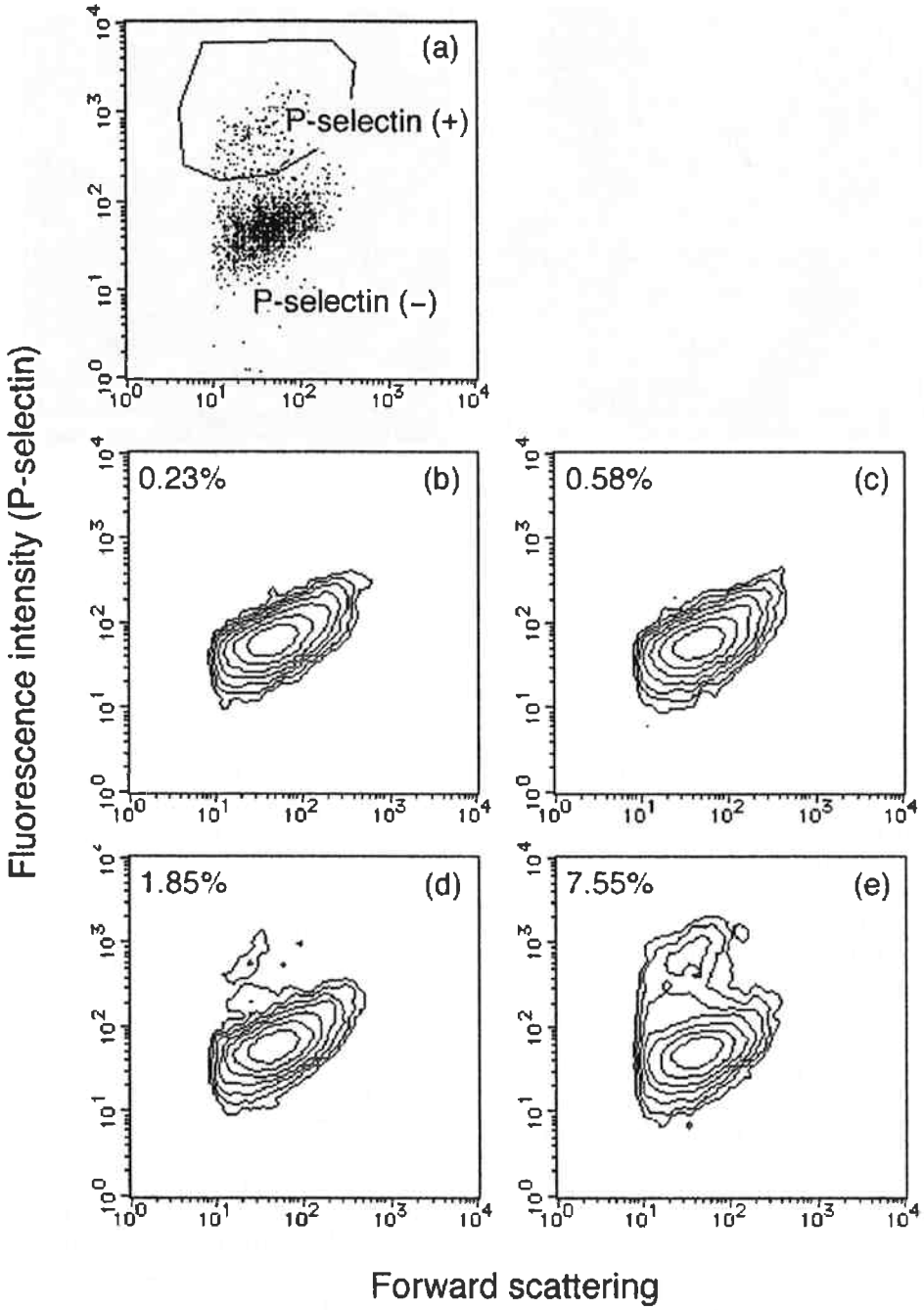

Figure 5. Flow cytometric analysis of the expression of P-selectin on the platelet surface. The platelets were incubated with hemodialysis membranes and were fixed with 1% formaldehyde, as described in Materials and Methods. The formaldehyde-fixed platelets were then stained with a combination of anti-P-selectin antibody and Alexa Fluor 488-conjugated anti-mouse IgG antibody, and were analyzed with a flow cytometer. The data are shown in a forward-scattering versus fluorescence intensity plot. The percentage of P-selectin-positive platelets (gated area in panel (a)) is indicated. (b), without membranes; (c), EVAL membranes; (d), PMMA membranes; and (e), PS membranes.

sion and activation than did hydrophilic membranes, such as those composed of RC or EVAL. The hydrophobic membranes also induced higher levels of platelet-mediated ROS production in neutrophils. These observations are in agreement with recent studies showing that modified PS membranes with increased hydrophilicity were less adhesive for platelets.[20,21] Based on these data, we postulate that the activation of platelets by their interaction with hemodialysis membranes contributes to the formation of P-selectin-mediated microaggregates and to subsequent ROS production in neutrophils. Because a relatively large membrane surface area was available for the contact with platelets in the present *in vitro* study, the phe-

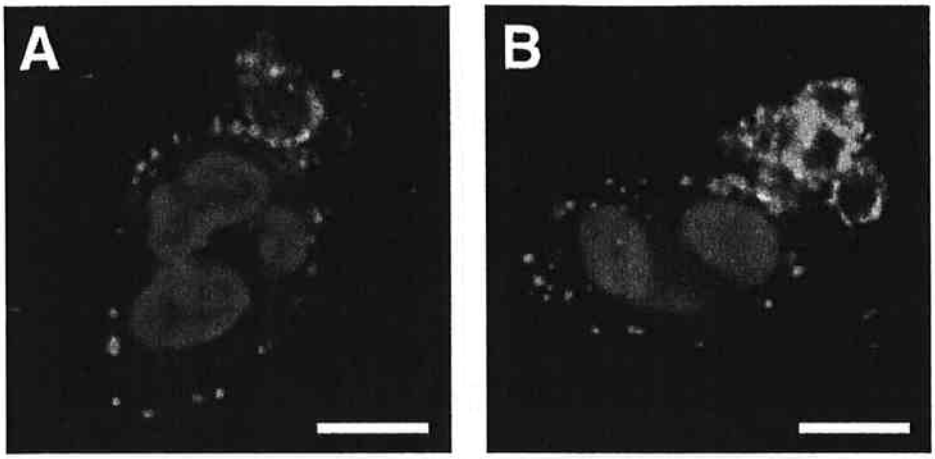

Figure 6. Formation of a platelet–neutrophil microaggregate after coincubation with hemodialysis membranes. PRP was incubated with PS (A) or PMMA (B) membranes at 37°C for 1 h. Platelets were then mixed with neutrophils, and the mixture was incubated at 37°C for 30 min. The cells were fixed with 1% formaldehyde for 10 min at room temperature. Activated platelets were stained with anti-P-selectin antibody (2D7) plus Alexa Fluor 488-conjugated anti-IgG (green). Neutrophils were stained with a combination of anti-sialyl LeX carbohydrate antibody (KM93), biotinylated anti-IgM antibody, and Alexa Fluor 555-conjugated streptavidin (red). The nuclei were stained with TO-PRO-3 (blue). The cells were observed with a confocal laser scanning microscope. Scale bar, 5 μm.

nomena observed may be exaggerated but are nonetheless likely to take place during hemodialysis. Although these processes seem to induce only low levels of ROS generation, the accumulation of slight damage to the vascular endothelium by ROS during repetitive hemodialytic therapy over the long term may cause hemodialysis-associated complications.[22,23] In patients with chronic renal failure, lowered levels of glutathione metabolism were reported to impair the elimination of ROS,[24,25] which may severely aggravate endothelial damage.

It has been suggested that complement activation is a major cause of intradialytic leukocyte activation.[26–28] Hydrophilic membranes, such as those composed of RC, were reported to have relatively high inducibility for complement activation,[29–31] although they had low inducibility for platelet activation, as shown in the present *in vitro* study. Complement activation and platelet–neutrophil coaggregate formation are interrelated, since C5a and C3a, generated by complement activation, induced neutrophils to express adhesion molecules and to promote platelet–neutrophil adhesion. Several reports have in fact shown that RC membranes induce the activation of platelets during hemodialysis.[32–34] The platelet activation by these membranes is possibly mediated by intricate processes involving complements and leukocytes. The inducibility for platelet activation as well as for complement activation seems to be an important factor in determining the biocompatibility of hemodialysis membranes.

The P-selectin-mediated pathway for leukocyte activation is also involved in the production of inflammatory cytokines and coagulation factors by leukocytes.[15,35–37] These physiologically active mediators are likely to evoke various types of inflammation- and coagulation-related adverse reactions that lead to hemodialysis-associated complications. The interaction between fibrinogen bound to activated platelets and integrin, such as CD11c/CD18, on neutrophils also plays a role in ROS production by neutrophils,[8] suggesting another platelet-mediated pathway for ROS production. A mechanism that is dependent on fibrinogen and integrin, as well as the P-selectin-mediated mechanism, may also be involved in platelet-mediated neutrophil activation during hemodialysis.

The platelet integrin αIIbβ3 (GPIIb/IIIa) has also been reported to be involved in the formation of the platelet–neutrophil complex mediated by fibrinogen.[18] The present study, however, showed that this integrin played a limited role in the platelet–neutrophil complex formation [Fig. 7(c)]. This apparent inconsistency might be explained by the presumption that platelet activation induced by hemodialysis membranes is rather moderate compared to that induced by physiologically potent stimulants such as thrombin, ADP, and collagen, and that the P-selectin-mediated adhesion precedes the integrin-mediated interactions under the conditions used in our *in vitro* study.

In conclusion, the interaction between platelets and hemodialysis membranes induced cascade reactions, including the cellular activation of platelets to express P-selectin on their surface, the formation of platelet–neutrophil microaggregates, and the production of ROS via P-selectin-mediated adhesion. The present study indicated that the platelet adhesion properties greatly affected the biocompatibility of hemodialysis membranes through these processes.

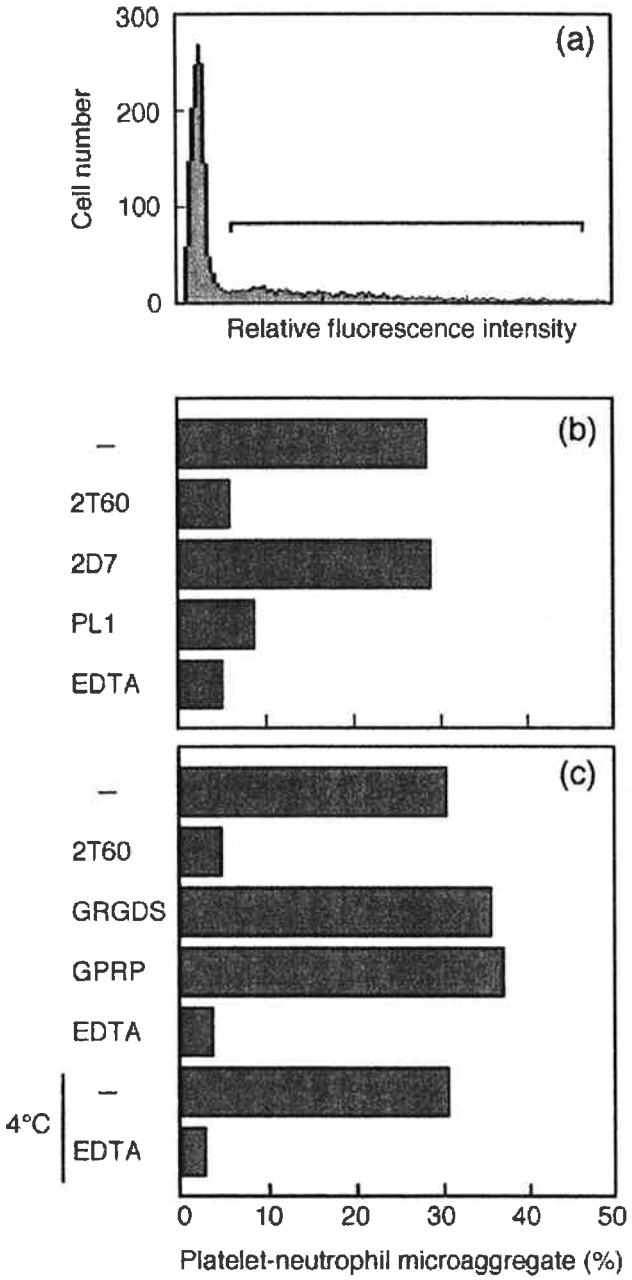

Figure 7. Flow cytometric analysis of the platelet–neutrophil microaggregates. Neutrophils were incubated with platelets that had been fluorescently labeled with BCECF at 37°C for 20 min, and the mixture was analyzed by flow cytometry, as described in Materials and Methods. Neutrophils were gated on a forward-scatter versus side-scatter plot, and their fluorescence profile is shown in a histogram (a). The fluorescence-positive neutrophils (indicated by a bar in panel (a)) were counted. The effects of antibodies and peptides on the microaggregate formation were examined (b and c). A representative result of three independent experiments is shown. 2T60, anti-P-selectin blocking antibody (20 μg/mL); 2D7, anti-P-selectin nonblocking antibody (20 μg/mL); PL1, anti-PSGL-1 antibody (20 μg/mL); EDTA, 5 m*M* EDTA; GRGDS, an integrin-recognizing peptide (2 m*M*); GPRP, a fibrin polymerization-inhibiting peptide (2 m*M*). The incubation was conducted at 4°C to minimize the integrin-mediated adhesion in some experiments.

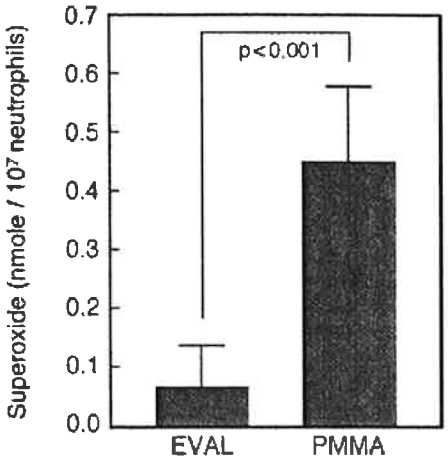

Figure 8. Production of superoxide anion by neutrophils after coincubation with platelets pretreated with hemodialysis membranes. The neutrophil suspension (1×10^6 cells, 50 μL) was mixed with a platelet suspension (50 μL) that had been treated with EVAL membranes or PMMA membranes, and the cells were incubated at 37°C for 90 min in the presence of cytochrome *c*. After centrifugation of the mixture at 5,000 rpm for 10 min, the supernatant was measured for A_{540}, A_{550}, and A_{560}. Superoxide anion was determined by the change in A_{550} caused by the reduction of cytochrome *c*.

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